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THE MOBILIZATION AND EXCRETION OF CALCIUM FOLLOWING OVERDOSAGE WITH IRRADIATED ERGOSTEROL.

N. B. TAYLOR AND C. B. WELD.

Department of Physiology, University of Toronto.

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IT has been quite positively established by many workers that irradiated ergosterol, when given in large doses to animals, will cause a marked hypercalcæmia. The mechanism by which the hypercalcæmia is produced is not, however, completely understood. There is dispute, indeed, over the important question of the source of the excess serum calcium.

Jones, Rapoport and Hodes (1930), Harris (1930), and Dixon and Hoyle (1928), have come to the conclusion that the excess serum calcium which follows ergosterol over-dosage is derived from the food, and that the sterol increases absorption from the gut. In a later communication Harris and Innes (1931) modify the original viewpoint, and consider that the excess calcium of the serum may be derived from the bones, provided that the dosage of irradiated ergosterol is sufficiently large and the amount of the ingested calcium is inadequate. Bills and Wirick (1930) have also shown that the toxic action of irradiated ergosterol is enhanced by increasing the amount of calcium in the food. It is not necessary, however, to conclude from this that increased absorption from the gut is responsible for the excess serum calcium. The effect of parathormone upon the serum calcium is also increased by high calcium diets, yet it has never been suggested that the hormone increases calcium absorption. In the face of the reports of many workers as to the effect of large doses of irradiated ergosterol in producing decalcification of the skeleton (Kreitmair and Hintzelmann, 1928; Branion and Kay, 1929; Light, Miller and Frey, 1929; Hess, Weinstock and Rivkin 1929; Baumgartner, King and Page, 1929; Brown and Shohl, 1930; György, 1930), the view that ergosterol increases calcium absorption must be carefully scrutinized.

The finding of Taylor, Weld, Branion and Kay (1931) that there is practically complete agreement between the morbid and metabolic changes following the administration of large doses of parathormone and of irradiated ergosterol, and the demonstration by several workers (Greenwald and Gross, 1925; Collip, 1926; Hunter and Aub, 1927; Stewart and Percival, 1927;

Bauer, Aub and Albright, 1929 ; Hunter, 1930) that large doses of parathyroid hormone cause decalcification of bone, becomes pertinent to the point at issue. Harris and Innes (1931) state that bone calcium is the sole source of the extra calcium resulting from parathormone administration, but draw a distinction between the action of the hormone and ergosterol when they conclude that the latter, but not the former, increases calcium absorption.

Jones, Rapoport and Hodes (1930), in upholding their hypothesis that the excess serum calcium is obtained only from food, criticize the experiments of Hess, Weinstock and Rivkin (1929), who showed that the serum calcium of rats on a diet deficient in calcium could be raised by irradiated ergosterol, on the grounds that even the small amounts of calcium present in the diet could be sufficient to account for the hypercalcæmia. They neglect the evidence of Light, Miller and Frey (1929), who demonstrated a hypercalcæmia in rats in negative calcium balance, following huge doses of irradiated ergosterol. The bones hold 95 per cent. or more of the body's calcium stores ; this fact, taken in conjunction with the observations cited above upon depletion of the bone calcium by irradiated ergosterol, points to bone as being the tissue involved in the production of the hypercalcæmia.

Jones *et al.* reported that hypercalcæmia could not be induced by the administration of irradiated ergosterol to dogs upon calcium-free diets. They considered that this fact indicated an exogenous source of the excess serum calcium resulting from ergosterol administration to animals upon ordinary diets. None of their animals, whether upon high or low calcium diets, showed any signs suggestive of a parathyroid response. In a preliminary report (1931) we showed that a very pronounced hypercalcæmia could be induced by ergosterol administration to dogs in negative calcium balance. It was then pointed out that the only interpretation of this result was that calcium had been mobilized from the body tissues, and that the conclusions of Jones *et al.* were unwarranted. In a recent paper Jones and Rapoport (1931) have receded from their original position, having found that by the employment of larger doses of the sterol a definite hypercalcæmia can be brought about in dogs upon calcium-free diets. In confirmation of the reports of Taylor, Branion and Kay (1930), and Taylor, Weld, Branion and Kay (1931), they have also reported the occurrence after ergosterol administration of the gastro-intestinal hæmorrhages, which we consider so characteristic of overdosage with parathormone.*

Much of the above work has been based upon experiments with rats. Rats and other species of rodents are known to withstand enormous doses of irradiated ergosterol and of parathormone (Taylor *et al.*, 1931). Most of our experiments have been carried out upon dogs, and we believe that these animals, both with regard to their calcium metabolism and in their reaction to vitamin D and parathormone, are more comparable to man. Rickets in dogs resembles the disease in infants. Wide variations in the Ca : P ratio may fail to induce it in either of the latter species, while it is generally conceded that rat rickets cannot be induced by vitamin D deficiency alone.

It is for this reason that we have chosen dogs as subjects for the investigation

* In a paper recently come to hand, Hess, Benjamin and Gross (1931) have reported similar findings.

of calcium metabolism. This investigation is still in progress, but certain of our results may now be assembled.

CALCIUM BALANCE EXPERIMENTS.

Methods.

The dogs were kept in metabolism cages, and total quantities of urine and fæces collected daily throughout the whole period of observation. This included a preliminary period of from 1 to 3 weeks on the diet planned for the experiment, during which time the normal level of serum calcium was determined. The duration of the preliminary period depended on the dog becoming accustomed to his new regimen and surroundings. The preliminary period continued until the urine and, if possible, the fæcal specimens were being excreted regularly and their calcium content had reached a fairly steady level. When this point was reached the administration of irradiated ergosterol was begun.* The serum calcium was determined by the Clarke and Collip (1925) modification of the Kramer and Tisdall (1921) method. The calcium of the food, fæces and urine was determined in the following way:

The whole 24-hour specimen of fæces, a representative sample of food, or a 100 c.c. of urine were dried in a platinum or silica dish and then ashed in an electric muffle furnace. The ash was then extracted by boiling with diluted nitric acid for about 5 minutes. Any undissolved residue was filtered off, and the solution neutralized (litmus paper) with strong ammonia and then re-acidified with 10 drops concentrated hydrochloric acid. Ten c.c. of 2·5 per cent. oxalic acid and 8 c.c. of 20 per cent. sodium acetate were then added and the solution shaken vigorously for 10 minutes. The precipitated calcium oxalate was then filtered off; washed on the paper once with 0·5 per cent. ammonium oxalate solution, and three times with water; and then perforating the paper it was washed off the paper with distilled water into a flask, where it was dissolved by adding 5 c.c. of concentrated sulphuric acid and made up to a volume of 100 c.c.

The whole solution (or an aliquot portion if the calcium precipitate had been very large) was then titrated while hot (60°–80° C.) with N/10 potassium permanganate solution until a faint permanent (1 minute) pink colour remained.

The N/10 $KMnO_4$ solution was checked by titrating against an accurately prepared N/10 sodium oxalate solution (in 0·5 per cent. H_2SO_4) at a temperature of 60°–80° C. Titrating in this way, 1 c.c. of N/10 potassium permanganate solution is equivalent to 2 mgm. calcium.

Table I shows a typical protocol, obtained in accordance with the above description.

This protocol indicates the method we have adopted in compiling the information given in Table II. In some cases urine and fæces were not excreted daily during the preliminary period, for which reason the later dogs were given infusorial earth daily in a fairly successful endeavour to obtain more

* This was very generously supplied by Mead, Johnson & Co., and was designated by them as having a potency of 10,000 *X* (see Bills *et al.*, 1931). This series of dogs was given 0·15 c.c. of the preparation per kilogramme of body-weight daily by mouth.

regular and frequent bowel movements. On the other hand, during the period of ergosterol administration the urine specimens (24 hours) were obtained regularly, though the fæcal specimens were even more infrequent.

TABLE I.—*Illustrating General Plan of Experiments. Dog* 41; *Weight*, 10 *kg.*

Day.	Serum calcium (mgm. per cent.).	Urine calcium (mgm.).	Fæces calcium (mgm.).	Food calcium (mgm.).
1	10·8	..	60·0	32
2	..	5·1	24·0	32
3	..	18·3	16·4	32
4	..	13·2	9·2	32
5	10·1	7·2	42·0	32
6	..	..	52·0	32
7	10·9	16·6	174·0	32
8	..	..	..	32
9	9·1	5·8	2·6	32
10	..	6·2	37·8	32
11	12·7	3·5	42·3	32
12	15·2	5·7	22·0	32
13	16·4	40·5	..	32
14	..	30·0	6·0	27·1
15	18·2	44·0	5·6	74·6
16	19·3	43·1	..	14·8
17	..	23·8	..	9·8
18	..	32·6	5·4	50
19	20·0	42·3 (F.)	..	50
20	16·6	54·0 (F.)	..	..

Preliminary period: Days 1–8.
Ergosterol period: Days 13–20 (ergosterol given daily after day 8).
(F.): Fæces and urine combined.
Serum calcium: Mgm. Ca per 100 c.c. serum; specimen taken on the morning of the day indicated.
Urine calcium: Mgm. Ca in specimen collected on the morning of the day indicated.
Food calcium: Mgm. Ca contained in the food actually eaten, which was given on the morning of the day indicated.

The Occurrence of Hypercalcæmia.

In considering the immediate point of discussion, namely, that hypercalcæmia may be obtained despite a negative calcium balance, the exact figures of this preliminary period are not of prime importance. As long as the dog can be said to be excreting a fairly constant amount of calcium by the end of the preliminary period, then the calcium equilibrium under the conditions of the experiment being known, a change in the balance may be ascribed to the administration of ergosterol. These factors will be discussed in more detail later.

In Table II the expression "ergosterol period" is used. This does not refer to the whole period during which the ergosterol is given, but only to that portion of it during which typical symptoms of overdosage are evident. The duration of this period is given in Table II, and the actual days selected are shown in the protocol recorded in Table I. In all cases there is a latent period following the giving of ergosterol before any effect can be noted, usually from 3 to 5 days. At the end of this time an abrupt change both in the calcium balance figures and in the health of the dog usually occurs and death may follow within 2 or 3 days. If the figures for the whole period which has elapsed since the beginning of the administration of ergosterol were averaged, the very decided effect which this material in large doses exerts upon the calcium

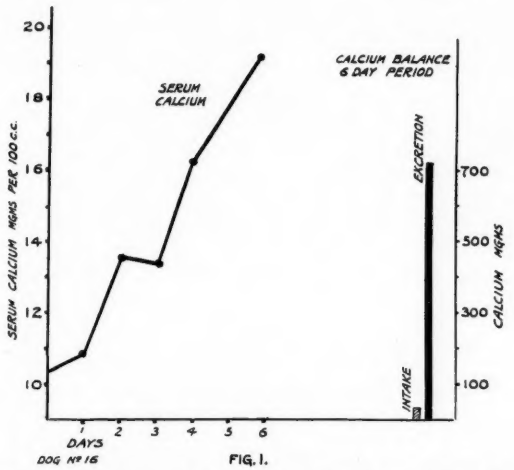

FIG. 1.—Showing hypercalcæmia of dog No. 16 during pronounced negative calcium balance.

balance would be more or less obscured. We have, therefore, selected those consecutive days during which hypercalcæmia, hypercalcuria and the general symptoms of overdosage were clearly evident, and grouped them under the heading "ergosterol period."

This table gives the results of calcium balance experiments upon 12 dogs. All were healthy adult animals, but of different ages and weights.

The earlier dogs of the series were fed with dried thyroid gland in order to induce artificially a negative calcium balance, but since it was found that irradiated ergosterol alone would cause a negative balance, the thyroid feeding was discontinued.

From Table II and Fig. 1 it will be seen that 11 of the 12 dogs showed negative calcium balances at the same time that the serum calcium was from 60 to 100 per cent. or more above the normal, and the total quantity of excess calcium in the serum amounted to from 9·8 to 126 mgm. It is apparent,

TABLE II.—*Calcium Balances and Serum Calcium after Administration of Irradiated Ergosterol.*

Dog No.	Weight (kg.).	Diet.		Preliminary period.		Ergosterol period.						
		Food.	Calcium (mgm.).	Length (days).	Serum calcium (mgm. per cent.).	Length (days).	Total calcium ingested (mgm.).	Total calcium excreted (mgm.).	Calcium balance (mgm.).	Highest serum calcium (mgm. per cent.).	Excess serum calcium. Mgm. per cent.	Excess serum calcium. Total mgm.
12	12·0	Meat, thyroid,* sugar	32·2	7	12·2	5	12·0*	221·6	−209·6	20·6	8·4	25·2
16	10·0	Ditto	17·0	7	10·7	6	51·0	713·1	−662·1	19·2	8·5	21·3
17	9·0	None	0·0	6	10·4	5	0·0	643·4	−643·4	20·4	10·0	22·5
24	6·7	Meat, sugar	1·6	14	10·4	8	3·2	474·7	−471·5	20·9	10·5	21·0
28	8·0	Ditto	8·0	11	12·0	12	8·0	1028·7	−1020·7	21·0	9·0	18·0
29	14·0	Ditto	8·0	13	10·3	5	32·0	84·0†	−36·0†	17·3	7·3	25·5
31	20·5	Biscuit	142·0	11	10·9	8	497·0	721·7	−224·7	31·0	20·1	126·0
32	14·0	Ditto	142·0	7	11·0	3	282·0	144·0	+138·0	21·0	10·0	35·0
33	4·0	Biscuit, infusorial earth	146·0	13	10·2	5	73·0	159·7	−86·7	20·0	9·8	9·8
34	7·6	Ditto	144·0	13	10·8	4	288·0	290·4	−2·4	17·5	6·7	12·7
40	6·9	Meat, biscuit, infusorial earth	32·0	9	10·6	6	312·0	587·9	−275·9	21·0	10·4	18·0
41	5·5	Ditto	32·0	8	10·5	8	258·3	440·3	−182·0	20·0	9·5	13·1

* Thyroid gland discontinued during period of ergosterol administration.
† Calcium of fæces not included.

then, that withdrawal of calcium from the tissues, rather than increased absorption from the bowel, is responsible for the hypercalcæmia of ergosterol administration. The column marked "excess serum calcium" refers to the total quantity of extra calcium in the animal's serum, and for calculation purposes we considered the blood to be one-twentieth of the body-weight and the volume of the serum to be half the blood volume.

Further discussion of the results obtained with these dogs, with special reference to the examination of the mechanism of the change in calcium metabolism, will be deferred until later in the paper. We wish first to present some results obtained with cats which have a more direct bearing upon the immediate subject of discussion, and which support our findings in dogs with regard to the source of the excess serum calcium.

EXPERIMENTS WITH CATS.

We have further evidence that irradiated ergosterol draws on the body tissues for its calcium for hypercalcæmia and excretion, from the following types of experiments. In the first series of experiments eviscerated cats were used, and it was found that in these the intravenous injection of irradiated ergosterol caused hypercalcæmia. In the second series, cats were poisoned with lead. The administration of irradiated ergosterol later to these animals caused an increased excretion of lead and calcium, though the bowel was practically free from lead at the time of the commencement of ergosterol feeding.

Eviscerated Cats.

Under ether anæsthesia the hepatic artery, portal vein and common bile-duct were ligated and the entire intestinal tract removed. Such an animal would live for several hours (from 4 to 8) when care was taken to give intravenous glucose after some 3 hours to avoid hypoglycæmic convulsions. Considered as a group these animals showed no significant increase in serum calcium. If, however, after obtaining a specimen of normal blood and eviscerating, 1 c.c. of 10,000 *X* irradiated ergosterol was given intravenously, a moderate hypercalcæmia was shown in many of the animals within 4 to 8 hours of the evisceration. The results of such a series of experiments are shown in Table III.

In view of the short duration of the experiments, negative results in some of the animals of the ergosterol group are scarcely significant. Since the reaction to irradiated ergosterol varies both in magnitude and time of onset in different individuals of the same species, it is to be expected that some animals would fail to respond during the short period of these experiments. It is seen that the greatest rise in serum calcium among the control group is 1·6 mgm. per 100 c.c. serum. Six of the 10 ergosterol animals show a greater rise than this. The average rise in the control animals is only 0·8 mgm., whereas the average rise in those of the ergosterol group is 2·6 mgm. The greatest rise in the latter group (when the premortal rise to 27·2 is excluded) is 7·8 mgm. In some experiments upon the action of parathormone carried

out previously, evisceration alone was found to exert no effect upon the serum calcium of cats. Stewart and Percival (1927) were also unable in their experiments to demonstrate that evisceration produced any significant rise in serum calcium. It seems justifiable to conclude that in cats, ergosterol is able to cause hypercalcæmia even in the absence of the intestinal tract. The excess calcium in the serum must, of necessity, be drawn from stores within the body under these circumstances.

TABLE III.—*Hypercalcæmia in Eviscerated Cats Following Administration of Large Doses of Irradiated Ergosterol.*

	Serum calcium (mgm. per cent.).		
	Before evisceration.	4–8 hours after evisceration.	
Control group	10·0	11·6	
,, ,,	10·0	11·4	
,, ,,	9·6	10·6	
,, ,,	12·7	13·3	
,, ,,	11·3	12·0	
,, ,,	10·8	10·2	
Average	10·7	11·5	Difference 0·8 mgm.
Ergosterol group	8·6	9·0	
,, ,,	10·3	13·2	
,, ,,	11·0	14·1	
,, ,,	10·0	11·9	
,, ,,	8·8	16·6	
,, ,,	10·2	11·4	
,, ,,	10·0	11·0	
,, ,,	12·9	18·4	
,, ,,	11·8	14·5*	
,, ,,	10·4	10·2	
Average	10·4	13·0	Difference 2·6 mgm.

* This was the value of the serum calcium one hour before death. Just prior to death the serum calcium was 27·2 mgm. per cent.

The Action of Irradiated Ergosterol upon the Excretion of Lead.

Having in mind the work of Hunter and Aub (1927) concerning the effect of parathormone upon the excretion of lead, we decided to determine whether or not irradiated ergosterol had an effect comparable to parathormone in ridding the body of lead. The results of these experiments have satisfied us that ergosterol definitely increases the excretion of lead in the urine. They also afford additional evidence concerning the source of the excess serum calcium. Hunter and Aub's experiments indicate that lead and calcium are deposited together in bone, and are apparently withdrawn together from the skeleton

and excreted in the urine under the influence of parathormone. Lead excretion may then, if these premises are correct, aid us in our efforts to trace the source of the excess serum calcium resulting from ergosterol overdosage.

Cats were given daily, for from 10–14 days, 50 mgm. of lead acetate in solution. The dose, except in the first two experiments,* was delivered by a syringe into the back of the mouth. The diet of all but two animals (9 and 10) consisted of raw meat alone. Animals 9 and 10 were given in addition a small amount of milk (50–75 c.c.) daily.

In six of the eight experiments in the series, a period of from 2–3 weeks was allowed to elapse before administering the ergosterol, in order to permit the daily excretion of lead by the bowel and kidney to reach a steady level. *The administration of large doses of irradiated ergosterol at the end of this time caused the excretion of lead in the urine to mount rapidly, and in some instances increase by several hundred per cent. The fæcal excretion of lead showed little change.* Two or three weeks seemed a sufficiently long time to allow for the ingested lead to become cleared from the intestinal tract. Furthermore, the amount of lead in the fæces at the end of this period was actually found to be very small. The appearance of large quantities of lead in the urine and the increase in urinary calcium under the influence of ergosterol therefore indicate, it seems to us, that the urinary lead and the associated calcium are derived from the bones rather than from the intestinal tract.

The doses of irradiated ergosterol given to these animals were large, 0·3 c.c. daily of 10,000 *X* per kilo. Within 4 or 5 days after the administration had been commenced the animals showed the usual symptoms of ergosterol (or of parathormone) overdosage, namely, somnolence, loss of appetite and diuresis. Death occurred usually within 4 or 5 days after the appearance of the first symptoms. When in similar experiments upon cats parathormone was substituted for the ergosterol, the results were practically identical.

TABLE IV.—*Excretion of Lead in the Urine and Fæces of Cats Before and After the Administration of Irradiated Ergosterol.*

Cat.	Preliminary period.			Effective ergosterol period.	
	Lead (mgm. daily average).		Calcium (mgm. daily average).	Lead (mgm. daily average).	Calcium (mgm. daily average).
	Urine.	Fæces.	Urine.	Urine.	Urine.
3	0·4	0·6	..	4·8	..
4	0·4	0·7	..	5·3	..
5	1·4	0·9	1·3	3·5	2·5
6	1·2	0·5	2·6	2·1	4·3
7	1·6	0·6	4·5	2·4	8·3
8	1·7	2·0	4·1	2·5	23·7
9	0·6	0·5	1·6	7·8	10·9
10	0·8	0·9	1·6	4·1	5·9

* In these two experiments the chemical was given in powder form. The taste of the material was evidently objectionable to the animals, and caused struggling and the most profuse salivation. After the first few administrations the animals gave a beautiful conditioned reflex response. Salivation and frothing at the mouth occurred when a stranger showed the animal, from a distance of several feet, a piece of paper folded into the shape of the powder paper.

FURTHER DISCUSSION OF EXPERIMENTS WITH DOGS.

Most of our dogs during the preliminary periods of the experiments showed negative calcium balances. Three animals (which were receiving larger amounts of calcium in their food) were in pronounced positive balance. This bears out the statement of Brown and Shohl (1930) that the amounts of calcium and phosphorus in the diet determine the retention of these elements. Bauer, Albright and Aub (1929) have also pointed out that if the calcium intake is low, it is exceeded in amount by the calcium of the urine and fæces. We have also observed that in starvation the daily excretion of calcium by the bowel alone may amount to several milligrammes. In short, a constant excretion through the intestine occurs independently of the calcium of the food.

TABLE V.—*Positive Calcium Balance Associated with a Normal Serum Calcium.*

Dog No.	Calcium daily.		Serum calcium (mgm. per cent.).
	Intake (mgm.).	Output (mgm.).	
32	142·0	15·6	11·0
33	144·0	20·3	10·2
34	144·0	41·7	10·8
24	1·6	39·0	10·4
28	8·0	26·0	12·0
29	8·0	96·6	10·3
31	142·0	204·6	10·9

During the preliminary periods the serum calcium of the animals which showed large positive balances was no higher than that of animals showing large negative balances (see Table V). Apparently the calcium is deposited too rapidly after absorption for hypercalcæmia to occur. Moreover, while it is possible to induce a positive balance by giving a calcium-rich diet, hypercalcæmia cannot be caused in this way. It is also true that the amount of calcium excreted in the urine was no less in animals showing positive balance than in those showing negative balances. It appears, then, that increased absorption, combined perhaps with reduced excretion through the intestine, is responsible for the positive calcium balance.

Harris and Innes (1931), though not denying that calcium is drawn from the bones as a result of ergosterol overdosage when the calcium available for absorption is small in amount, believe that when the ingested calcium is adequate the hypercalcæmia is the result of increased absorption. Why, then, does the greater absorption which occurs in the animals mentioned above cause no rise in serum calcium nor any unusual loss of calcium in the urine, whereas in overdosage with irradiated ergosterol, hypercalcæmia, hypercalcuria and a negative calcium balance go hand in hand? The mechanisms involved in the two circumstances are apparently quite different.

For these and other reasons which will be discussed later, we have been led to believe that the greater absorption which the reduction in fæcal calcium has been assumed to indicate is only apparent, and that it may be held more

reasonably to be due to reduced excretion into the intestine. Furthermore, when calcium is injected intravenously it disappears from the blood very quickly, yet only small amounts of the excess are got rid of through the kidneys and bowel. It is evidently stored within the body. Why, then, should the extra serum calcium resulting from ergosterol over-dosage—if it be derived from the intestinal tract—be excreted again in such large quantities in the urine ? If, on the other hand, the excess calcium comes from bone, the loss of calcium in the urine becomes intelligible.

The protocol of dog 32 is given in Table VI. This animal is the only one of our series which showed a positive balance during the administration of ergosterol as well as during the preliminary period. The fall in fæcal calcium and the rise in urinary calcium as the animal came under the influence of ergosterol are clearly shown. It should be noted that the fæcal calcium first showed a decided fall, and that the serum calcium became elevated before a rise in urinary calcium occurred.

TABLE VI.—*Protocol Showing the Order of Appearance of Reduction in Fœcal Calcium, Hypercalcæmia and Hypercalcuria. Dog.* 32, *Female,* 14 *kg.*

Day.	Serum calcium (mgm. per cent.).	Urine calcium (mgm.).	Fæces calcium (mgm.).	Food calcium (mgm.).	Irradiated ergosterol 10,000 *X*.	Remarks.
1	..	8·8	..	142	..	..
2	10·8	3·0	..	142	..	..
3	..	4·0	58·7	142	..	..
4	..	4·2	12·2	142	..	..
5	..	2·4	..	142	..	..
6	..	1·8	..	142	..	..
7	11·0	3·6	10·0	142	2·0 c.c.	..
8	10·8	2·0	..	142	2·0 ,,	..
9	..	2·2	1·5	142	2·0 ,,	..
10	12·5	3·3	..	142	2·0 ,,	..
11	..	15·0	5·0	142	2·0 ,,	Symptoms of overdosage.
12	21·0	54·0	4·6	142	2·0 ,,	..
13	..	64·4	..	..	2·0 ,,	Death.

The ergosterol was given and the serum calcium obtained during the morning of the day shown. The excretion and food figures represent the amount of calcium excreted or ingested for the period ending on the morning of the day shown.

One would suppose that had the preliminary periods of dogs 32, 33 and 34 (Table V) been prolonged for several weeks, the excretion of calcium would have risen and brought the animal into calcium equilibrium. As against this, however, Hart, Steenbock and Elvehjem (1924) recorded a continuous positive calcium balance in a goat for nine months on a diet containing only a moderate excess of calcium. In fact, they could not bring the animal into the equilibrium or the negative balance which they desired until the calcium intake had been reduced.

It would seem from these observations that for a period of a few weeks at

least one should be able to obtain a positive or a negative calcium balance at will, merely by raising or lowering the calcium content of the diet. Our results are in accordance with this, as will be seen from Table VII, where reference to the "preliminary period" columns will show no correlation between the intake and output of calcium. This table has been prepared more particularly, however, for use in discussion of the change in calcium balance which occurs following the administration of irradiated ergosterol in toxic doses.

TABLE VII.—*Calcium Balances.*

Dog No.	Total calcium intake (daily average in mgm.).		Total calcium output (daily average in mgm.).	
	Preliminary period.	Ergosterol period.	Preliminary period.	Ergosterol period.
17	Starving	Starving	Not done	107·4
24	1·6	0·4	39·0	59·4
28	8·0	0·7	26·0	83·9
29	8·0	6·4	96·6	72·8
31	142·0	62·1	204·6	90·2
32	142·0	94·0	15·6	47·7
33	146·0	0	20·3	31·9
34	144·0	72·0	41·7	74·6
40	32·0	52·0	[illegible]·0	97·3
41	32·0	32·0	53·6	40·9

Examination of the figures in Table VII reveals that in 6 cases out of 9 the total excretion of calcium is increased under the influence of ergosterol. This occurs despite, in most instances, the refusal of food, and as a result of this a lowered calcium intake and a reduced calcium balance. It is true that the lessened calcium intake without any change in excretion could in some cases have resulted in a negative balance, but an increase in calcium excretion in the face of a reduced intake certainly indicates the mobilization of calcium from stores within the body.

The Routes through which Calcium is Normally Excreted from the Body, and the Effect thereon of Ergosterol Administration.

Greenwald and Gross (1929) in their experiments found that in normal dogs only a very small proportion of the total calcium excretion occurred through the urine. We have obtained similar results which are shown in Table VIII. Under the influence of ergosterol, however, as this table shows, the excretion was switched very strikingly from the fæcal to the urinary route. This shift of calcium from fæces to urine has been remarked upon by Brown and Shohl (1930), who observed it in rats. The relative amounts of calcium in urine and fæces before and after the administration of ergosterol are shown graphically in Fig. 2. The increase in the total amount of calcium excreted after ergosterol administration is also clearly shown in Tables VII and VIII.

A Comparison of the Effects upon Calcium Metabolism of Thyroid and Irradiated Ergosterol.

Though thyroid feeding causes decalcification of the bones and increased calcium excretion, a rise in serum calcium does not occur, and the mobilized calcium is passed in the fæces as well as in the urine. According to Aub, Bauer, Heath and Ropes (1929), in man the excreted calcium is about equally partitioned between the urine and fæces, yet in the few animals to which we have fed thyroid, a much greater proportion of the calcium loss occurred in the

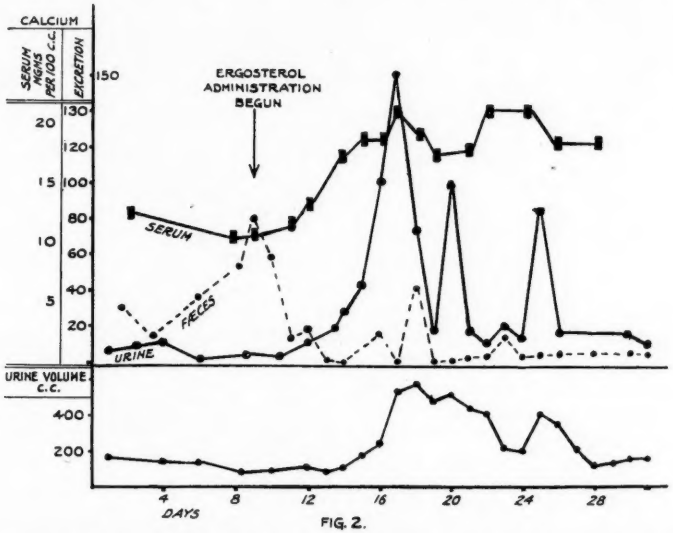

FIG. 2.

FIG. 2.—Curves of serum calcium, fæcal and urinary calcium, and urinary volume. The fæcal calcium of this dog (No. 40) was relatively low during the first half of the preliminary period. In other animals the fæcal calcium was usually considerably higher at this time, and in consequence the fall resulting from ergosterol administration was more decisive.

fæces. The excretion of calcium in the urine was only moderately increased, whereas the loss of calcium in the fæces was enormous, amounting in one instance to nearly 500 mgm. during a three-day period, though the animal received during this time only 51 mgm. in his food. Upon administering irradiated ergosterol to this animal and continuing the thyroid feeding, the fæcal excretion of calcium was promptly reduced, whereas the urinary calcium became increased by 90 per cent. It is evident that the manner by which the body disposes of the calcium released from the bones is not the same after thyroid feeding as after ergosterol overdosage. In all our animals under the influence of the latter substance, there occurred a reduction in the calcium of the fæces followed by a rise in serum calcium, and finally a rise in urinary calcium (Table VIII). This order was the rule. It would appear, then, that

TABLE VIII.—*Routes of Calcium Excretion in Normal Dogs Compared with those Receiving Ergosterol.*

Dog No.	Preliminary period.		Ergosterol period.	
	Urine (daily average mgm.).	Fæces (daily average mgm.).	Urine (daily average mgm.).	Fæces (daily average mgm.).
17	Not determined	Not determined	78·0	29·4
24	1·7	37·3	51·5	7·9
28	5·6	20·4	76·9	7·0
29	13·5	83·1	16·8	56·0*
31	4·6	200·0	89·7	0·5
32	4·0	11·6	44·5	3·2
33	4·6	15·7	23·7	8·2
34	13·0	28·7	48·0	26·6
40	7·0	37·0	86·0	11·3
41	6·6	47·0	38·8	2·1

* Only one specimen of fæces obtained. It was on the last day, but the average is of doubtful significance.

when as a result of ergosterol overdosage the calcium enters the blood from the bones, the threshold is raised against the excretion of the mineral through the bowel. Not until the serum calcium has become elevated quite definitely does it overflow into the urine—the level of the serum calcium at which the threshold of the kidney was crossed varied in different animals (see Table IX).

TABLE IX.—*Renal Threshold for Calcium in Dogs Receiving Large Doses of Irradiated Ergosterol.*

Day.	Dog 39.		Dog 40.		Dog 32.	
	Serum calcium.*	Urine calcium.†	Serum calcium.*	Urine calcium.†	Serum calcium.*	Urine calcium.†
1	10·6	5·8	10·6	11·2	11·0	3·6
2	..	6·2	9·5	..	10·8	2·0
3	12·7	3·5	11·8	9·2‡	..	2·2
4	15·2	5·7	13·1	10·3	12·5	3·3
5	16·4	40·5	..	..	..	15·0
6	..	30·0	17·3	17·6‡	21·0	54·0
7	18·2	44·0	17·8	43·2	..	64·0
8	19·3	43·0	21·0	156·0	..	..

* Serum calcium, expressed as mgm. per 100 c.c. serum; specimen taken on the morning of the day shown.

† Urine calcium (mgm.) in the 24-hour specimen of urine collected on the morning of the day shown.

‡ Calcium (mgm.) contained in the 48-hour specimen of urine.

The Calcium of the Fæces.

The quantity of calcium in the fæces is usually assumed to be the sum of that excreted into the bowel and of that unabsorbed from the food. Except

in experiments of short duration, it is impossible to determine by direct means each of these fractions separately. Such short-term experiments have shown, indeed, that the amount of calcium excreted through the small and large bowel is not great (Percival and Stewart, 1927 ; Taylor and Fine, 1930). But in these experiments, in which the bowel was flushed through before and after a period of from 3 to 6 hours, the animals cannot be reasonably considered as normal. On the other hand, we may, by a study of the fæcal calcium in intact animals, gain indirectly some valuable information concerning the relative contributions which excreted and non-absorbed calcium make toward the sum total of the mineral which leaves the body by the bowel.

If the non-absorbed fraction contributed greatly to the fæcal calcium, we should expect that those dogs of our series which received larger amounts of calcium in the food would show higher figures for fæcal calcium than the dogs receiving smaller amounts. Table X, which is made up of the figures obtained during the preliminary periods of our dogs, shows no such correlation.

TABLE X.—*Showing Lack of Correlation between Ingested and Fæcal Calcium.*

Dog No.	Calcium ingested (mgm. daily).	Fæcal calcium (mgm. daily).	Total period of experiment (days).
24	1·6	43·2	14
28	8·0	20·4	11
29	8·0	90·1	11
40	32·0	28·8	9
41	32·0	47·2	8
45	32·0	10·0	16
53	32·0	16·4	20
46	45·0	9·6	12
47	49·5	14·7	21
54	62·0	13·0	20
54	Starvation	12·0	6
31	142·0	200·0	6
32	142·0	11·7	7
33	146·0	15·9	13
34	144·0	31·1	12

The lack of agreement between the figures of the two columns leads one to believe that under the conditions of our experiments the ingested calcium is all absorbed, and that the calcium of the fæces is composed practically entirely of excreted calcium.

To investigate this problem further, experiments of the following type were undertaken : Extra calcium in the form of calcium chloride solution was given by mouth daily to a dog after the figures for its fæcal calcium had become fairly constant. Determinations were then made to show the proportion of this added calcium which could be recovered from the fæces. The following is an account of the results obtained so far : In the first experiment 50 mgm. of calcium were administered upon two successive days. Of this, 60 per cent. appeared in the fæces during the three days following the first

day's administration. The fæcal calcium then fell to its previous level. After the lapse of two days a second dose of 50 mgm. was given. This was recovered practically quantitatively the following day. Subsequent daily doses—four in all of 50 mgm. each—were, however, without effect upon the calcium content of the fæces. The daily average of this for the ten days during which the extra calcium was given was actually lower than for the preliminary period during which the animal had received an ordinary diet containing 32 mgm. per daily ration. A second normal dog (dog. 45, Table XI) reacted in practically the same way when calcium was added to the diet. Of the first three doses, about 55 per cent. was recovered within 4 days following the first day of the administration. After this time the calcium added upon three successive days was completely absorbed. These results suggested to us that *the calcium in an ordinary diet is completely absorbed, and the calcium of the fæces represents that which has been excreted through the intestinal mucosa.* The quantity of calcium passed in the fæces of the animals upon these calcium-rich diets was no greater, indeed considerably less, than that of some animals during starvation or upon calcium-deficient diets. In these latter the fæcal calcium certainly must be excreted calcium, so there is little reason to doubt that the fæcal calcium of the former animals was also excreted and not unabsorbed calcium.

Following upon this conclusion came the question, How, then, is it possible for the absorption of calcium, already complete even in an animal on a high calcium intake, to be improved by ergosterol administration ? An experiment on the same plan as those described above was carried out upon an animal (No. 40, Table XI) under the influence of ergosterol. The calcium of the fæces was affected after very much the same fashion in this animal as in animal 45. Fifty per cent. of the first two daily doses was found in the fæces during the two days following the first dose, and the output in the fæces then returned to its previous level. This experiment failed to demonstrate that ergosterol increased calcium absorption.

Three other normal animals (Nos. 47, 53, 55, Table XI) were given extra calcium over a longer period than animals 45 and 40. But, as in the latter, the calcium excretion was found to be no greater during the period of extra calcium feeding than during the preliminary period. No significant rise in serum calcium or in the calcium excreted in the urine occurred. The extra calcium was therefore deposited within the body—probably in the bones. We are continuing to feed these animals with calcium in excess, the daily dose being gradually increased. It is inevitable that calcium retention will reach a limit if the feeding is continued with increasing doses. It is likely that this limit will be indicated by a rise in the fæcal calcium approaching in amount the calcium of the diet.

In Table XI the excretion of calcium over a period of several weeks is shown. These dogs were first kept upon a normal diet containing 32 mgm. of calcium. They were then given extra calcium for 6 or 7 consecutive days and the excretion followed for a similar period thereafter. Dogs 53 and 55 were subsequently starved for a corresponding period. It will be noted that the average daily calcium excretion is practically the same during all three periods. These results are at variance with those of Telfer (1922), who reported that in infants the quantity of calcium excreted in the fæces was proportional to the amount

of calcium ingested. A large proportion of Telfer's experiments showed a negative calcium balance or a small positive balance. It is difficult to understand how such balances could continue for any length of time in a healthy,

TABLE XI.—*Fate of Ingested Calcium. Part I.*

Day.	Dog 45. Normal. Diet contains 32 mgm. calcium daily.			Dog 40. Treated with ergosterol. Average daily diet contains 32 mgm. calcium.		
	Extra ingested calcium (mgm.).	Urine calcium (mgm.).	Fæces calcium (mgm.).	Extra ingested calcium (mgm.).	Urine calcium (mgm).	Fæces calcium (mgm.).
1	0	8·5	..	0	..	6·4
2	0	14·6	13·2	0	17·6	2·6
3	0	10·0	..	0	43·2	2·2
4	0	7·8	3·2	45	113·4	14·0
5	0	..	6·0	45	156·0	2·5
6	50	21·6	3·6	45	77·0	44·0
7	50	11·5	4·0	0	15·3	2·4
8	50	22·8	30·0	0	111·0	2·4
9	0	11·5	4·0	0	15·9	2·4
10	50	19·2	72·0	45	10·4	..
11	50	8·1	2·4	45	23·4	18·8
12	50	8·5	3·4	0	12·0	5·0
13	0	9·1	3·8	0	84·6	6·0
14	0	12·5	..	0	16·6	5·4
15	0	9·5	7·6	..	..	..
16	0	..	..	..	..	..
17	0	16·1	3·4	..	..	..

Extra ingested calcium given orally by syringe on morning of day noted.
Urine and fæces.—Specimens collected on morning of day noted.

Part II.

Dog No.	Diet calcium (mgm. daily).	Preliminary period.			Extra calcium period.*			Starvation period.†		
		Days.	Urine calcium (mgm.).	Fæces calcium (mgm.).	Days.	Urine calcium (mgm.).	Fæces calcium (mgm.).	Days.	Urine calcium (mgm.).	Fæces calcium (mgm.).
47	32	7	7·3	14·5	14	5·7	16·1	Not starved.		
53	36	29	8·3	18·9	12	7·5	18·4	12	15·9	15·0
55	36	30	14·1	8·8	12	8·1	9·0	12	12·2	8·0

* 50 mgm. calcium in form of calcium chloride solution given daily by mouth for first one half of days shown. Collections continued over whole period to ensure excretion of any calcium remaining in intestinal tract.

† No food given to dogs, for first one half of days shown. Calcium-free diet fed on remaining days.

growing infant. Telfer also states that in his experiments there was no evidence that any of the fæcal calcium had an endogenous origin. Our results are more in agreement with those of Sherman and Hawley (1922), who found that in children there was no close relationship between the quantities of

calcium ingested and excreted. The amount of calcium excreted upon a low calcium intake was frequently as high as, or higher, than when the intake was much greater. The absorption increased with the intake until this had reached a level of between 1 and 2 gm. per day.

SUMMARY.

1. Large doses of irradiated ergosterol when administered to dogs in negative calcium balance cause hypercalcæmia. The total calcium excretion is also increased. The experiments indicate that the excess calcium in the serum is derived from body tissues.

2. Hypercalcæmia has been produced by the intravenous administration of irradiated ergosterol to cats from which the gastro-intestinal tract had been removed.

3. Cats poisoned with lead and given large doses of ergosterol after the lead administration had been discontinued showed a striking increase in the quantity of the metal excreted in the urine. Large quantities of calcium were excreted with the lead.

4. In normal dogs calcium is excreted chiefly by the bowel; only small quantities appear in the urine. Under the influence of large doses of irradiated ergosterol the route for the excretion of calcium is turned from the intestine to the kidney. Evidence is submitted in support of the conclusion that the reduction in fæcal calcium which follows ergosterol administration in this dosage is due to depression of the intestinal excretion rather than, as has been supposed, to increased absorption.

5. No correlation between the calcium content of the diet and the quantity of calcium in the fæces could be found. Animals during starvation excreted as much calcium in the fæces as those upon diets containing moderate quantities of calcium. The amount of calcium excreted by animals upon a normal diet was just as great as when, during later periods, they received excess calcium in the form of calcium chloride.

CONCLUSIONS.

The hypercalcæmia following large doses of irradiated ergosterol is due to the withdrawal of calcium from the skeleton. Ergosterol overdosage depresses the power of the intestine to excrete calcium. The calcium mobilized from the bones accumulates in the serum. A certain level of serum calcium having been reached, an increase in the urinary calcium follows. Ergosterol in the amounts which produce hypercalcæmia does not increase absorption of calcium from the intestine.

REFERENCES.

Aub, J. C., Bauer, W., Heath, C., and Ropes, M.—(1929) *J. Clin. Invest.*, **7**, 97.
Bauer, W., Albright, F., and Aub, J. C.—(1929) *Ibid.*, **7**, 75.
Bauer, W., Aub, J. C., and Albright, F.—(1929) *J. Exp. Med.*, **49**, 145.
Baumgartner, L., King, E. J., and Page, I. H.—(1929) *Biochem. Z.*, **213**, 170.
Bills, C. E., and Wirick, A. M.—(1930) *J. Biol. Chem.*, **86**, 117.
Bills, C. E., Honeywell, E. M., Wirick, A. M., and Nussmeier, M.—(1930) *Ibid.*, **90**, 619.

BRANION, H. D., AND KAY, H. D.—*Unpublished.*
BROWN, H. B., AND SHOHL, H. T.—(1930) *J. Biol. Chem.*, **86**, 245.
CLARKE, E. P., AND COLLIP, J. B.—(1925) *Ibid.*, **63**, 461.
COLLIP, J. B.—(1926) *Medicine*, **5**, 1.
DIXON, W. E., AND HOYLE, J. C.—(1928) *Brit. Med. J.*, **2**, 832.
GREENWALD, I., AND GROSS, J.—(1925) *J. Biol. Chem.*, **66**, 217; (1929) *ibid.*, **82**, 505.
GYÖRGY, P.—(1930) *Klin. Wochenschr.*, **9**, 102.
HARRIS, L. J.—(1930) *Lancet*, **1**, 236.
Idem AND INNES, J. R. M.—(1931) *Biochem. J.*, **25**, 367.
HART, E. B., STEENBOCK, H., AND ELVEHJEM, C. A.—(1924) *J. Biol. Chem.*, **62**, 117.
HESS, A. F., BENJAMIN, H. R., AND GROSS, J.—(1931) *Ibid.*, **94**, 1.
HESS, A. F., WEINSTOCK, M., AND RIVKIN, H.—(1929) *Proc. Soc. Exp. Biol. Med.*, **26**, 199.
HUNTER, D.—(1930) *Lancet*, **1**, 879, 974, 999.
Idem AND AUB, J. C.—(1927) *Quart. J. Med.*, **20**, 123.
JONES, J. H., AND RAPOPORT, M.—(1931) *J. Biol. Chem.*, **93**, 153.
JONES, J. H., RAPOPORT, M., AND HODES, H. L.—(1930) *Ibid.*, **89**, 647.
KRAMER, B., AND TISDALL, F. F.—(1921) *Ibid.*, **47**, 475.
KREITMAIR, H., AND HINTZELMANN, U.—(1928) *Arch. exp. Path. Pharm.*, **137**, 203.
LIGHT, R. F., MILLER, G., AND FREY, C. N.—(1929) *J. Biol. Chem.*, **84**, 487.
SHERMAN, H. C., AND HAWLEY, E.—(1922) *Ibid.*, **53**, 375.
STEWART, C. P., AND PERCIVAL, G. H.—(1927) *Biochem. J.*, **21**, 301.
TAYLOR, N. B., AND FINE, A.—(1930) *Amer. J. Physiol.*, **93**, 544.
TAYLOR, N. B., AND WELD, C. B.—(1931) *Ibid.*, **97**, 566.
TAYLOR, N. B., BRANION, H. D., AND KAY, H. D.—(1930) *J. Physiol.*, **69**, xxxv.
TAYLOR, N. B., WELD, C. B., BRANION, H. D., AND KAY, H. D.—(1931) *Can. Med. Ass. J.*, **24**, 763; (1931) *ibid.*, **25**, 20.
TELFER, S. V.—(1922) *Quart. J. Med.*, **16**, 45.

A DISPLACEMENT METHOD FOR ESTIMATING THE CELL-VOLUME IN BLOOD.

C. E. JENKINS.

From the Pathological Department, Royal Hospital, Salford.

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THE accuracy of the hæmatocrit method for estimating cell-volume in blood rests upon the assumption, that the centrifugal pressure applied is sufficient to change the shape of the cells to such an extent that the whole of the intercellular space is obliterated. The only force acting is centrifugal, and that is proportionate to the density of the individual cell relative to the density of the surrounding fluid.

A cell in the top layer of the deposit is influenced only by its own weight pressing it against the cell next below. But a cell at the bottom is compressed not only by its own weight, but also by the pressure of the column of cells above. For this reason it is improbable that the colour of the mass will be the same throughout if the lake criterion has any definite value.

It is, on the other hand, probable that the spaces will not be obliterated in

the upper part of the column, whatever the pattern of tube used. Jorgensen and Warburg (1927) stress the importance of centrifugalizing to a lake colour, and from an inspection of the three colours called lake presumably crimson lake is meant. A thick smear of this was, therefore, used as a standard, and every hæmatocrit reading was compared with it. I found that the top of the column matched the paint, the middle was slightly darker, and the bottom was decidedly darker ; this gradation in colour is what one would expect from the theoretical considerations. The speed of the centrifuge was estimated to be about 5200 r.p.m., and the cell volume became constant in 40 minutes. A period of 1 hour was standardized for all hæmatocrit estimations.

Oxalated blood was used. Osgood (1926) states that 0·2 per cent. oxalate contracts the corpuscles by 3·5 per cent., so every result in this paper has had that value added to it.

In the displacement method, the principle is to replace a known percentage of clear plasma with the same volume of autogenous hæmolyzed plasma, and to measure the resulting change in the hæmoglobin concentration and to compare it with a standard. By using autogenous hæmolyzed plasma one obtained a non-diffusible colloid, which can be measured by its depth of colour, and as the osmotic pressure must obviously be unchanged the cells do not alter in size.

The method is as follows : About 10 c.c. of blood is taken from a vein, and shaken with sufficient potassium oxalate to make a 0·2 per cent. solution. A hæmatocrit tube is filled and centrifugalized. Whilst it is spinning, about 3 or 4 c.c. of blood is frozen solid in a mixture of ice and rock salt. Solidification is complete when a raised peak is seen on the surface of the frozen mass. The blood is next thawed by immersion in water at 37° C. and the freezing process started again. The freezing and thawing is done five times in all and takes about 45 minutes. Oxalate plays no part in the hæmolysis, because blood free from any anticoagulant can be hæmolyzed with equal ease, clotting being prevented by the low temperature. A cell-count is also done.

The proportion of cells hæmolyzed can be ascertained by putting a drop of the stained plasma before centrifugalization, and undiluted, into a counting chamber. The recognizable cells have varied from 2 in one small square to 1 in five small squares. Calculation will show that the proportion hæmolyzed varies from 99·8 to 99·98 per cent.

If hæmoglobin estimations of the stained plasma before and after centrifugalization be made, it will be found that the centrifugalized specimen is about 2 or 3 per cent. higher than the other, because the latter is made up of the stained plasma and the solid particles in suspension, which must displace their own volume of plasma, whereas the centrifugalized specimen contains nothing but hæmolyzed plasma. It is an interesting confirmation of a suggestion made by Don and myself (1931), concerning the cause of an error that we detected in the Van Slyke method (1921) when comparing it with the flicker hæmoglobinometer.

At the end of the hour the hæmatocrit reading is taken, and the tube set aside whilst the hæmolyzed plasma is spun to remove the cell *débris*, which is colourless and small in amount. The clear plasma from the hæmatocrit tube is removed until the meniscus is at exactly 50 per cent., and the hæmolyzed plasma added until the meniscus is back at 100 per cent. again. The stained

plasma is thus diluted by the plasma that remains in the tube above and between the cells. The tube is shaken very thoroughly to ensure even diffusion, and is then centrifugalized. Meanwhile the standard is prepared by adding 0·1 c.c. of hæmolyzed plasma to 9·9 c.c. of distilled water, and the solution prepared for examination by the flicker hæmoglobinometer. This instrument is used to obtain the greatest possible accuracy, but a colorimeter can be substituted; the increased error will not be large enough to vitiate the results. The tube of blood is spun for about 7 minutes, which is enough to give a fairly wide zone of stained plasma free from cells. About 0·5 c.c. is withdrawn, and 0·1 c.c. is added to 4·9 c.c. of distilled water and prepared in the same way as the other sample.

The standard dilution of 1 in 100 is equivalent to a dilution of stained plasma that has previously been diluted with its own volume of colourless plasma. The formula for calculating the percentage cell volume of the blood then is—

$$\frac{\text{(Strength of tube sample)} - \text{(strength of standard)}}{\text{(Strength of tube sample.)}}$$

The following ten results are consecutive and taken at random, so are not representative of the normal mean, but are sufficient to show the difference between the two methods:

TABLE.—*Comparison of Displacement and Hæmatocrit Methods. The Volumes are Expressed as Percentages, and are Calculated to the Basis of 5 Million Cells.*

Displacement.	Hæmatocrit.	Difference.
39·0	42·2	8·2
32·7	37·0	13·1
39·4	42·4	7·6
36·3	42·4	16·7
35·1	40·1	14·2
48·3	53·6	10·9
33·0	36·9	11·7
33·6	37·4	11·4
38·3	42·2	10·2
32·7	41·4	26·6 (*)
Mean of first nine:		
37·3	41·6	11·5

The tenth result, showing a very large difference, is an artefact produced by mixing two samples of blood. The colour test of the hæmatocrit showed that the mass had not packed, and it refused to do so on further centrifugalization, although another sample spun at the same time behaved normally. The stated difference is, therefore, supported by this fact, and I was at a loss to explain it until I realized that if the bloods belonged to different groups clumping would occur, and the large masses could not be expected to pack as closely as discrete cells. The remainder of the mixture was examined and found to be full of clumps. The result demonstrates that the packing of cells

can be influenced by external factors, as well as by the flexibility of their interiors. It also suggests that blood showing auto-hæmagglutination might also resist packing, and give an erroneous result by the hæmatocrit method.

The table shows that the hæmatocrit obliterates most of the intercellular space, but it does not obliterate the whole of it.

SUMMARY.

A method for estimating the volume of the corpuscles in blood is described. The volume so found is smaller than that found by the hæmatocrit method.

REFERENCES.

JENKINS, C. E., AND DON, C. S. D.—(1931) *Brit. J. Exp. Path.*, **12**, 212.
JORGENSEN, S., AND WARBURG, E. J.—(1927) *Act. Med. Scand.*, **16**, 468.
OSGOOD, E. E.—(1926) *Arch. Intern. Med.*, **37**, 685.
VAN SLYKE, D. D., AND STADIE, W. C.—(1921) *J. Biol. Chem.*, **49**, 1.

OBSERVATIONS ON THE INORGANIC SULPHATE CONTENT OF THE BLOOD IN ECLAMPSIA.

DAVID FYFE ANDERSON AND SIDNEY LIONEL TOMPSETT.

From the Royal Maternity and Women's Hospital, and the Biochemical Laboratory, Royal Infirmary, Glasgow.

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COMPARATIVELY few observations have been made on the inorganic sulphate content of human blood. From a perusal of the literature, this would appear to be mainly due to the difficulties experienced in the determination of the low concentrations present in the blood. In a multiplicity of investigations on the more common constituents of the blood in eclampsia, inorganic sulphate seems to have been neglected. Any finding having a possible relationship to the origin of eclampsia is of prime importance, hence our decision to place on record the results obtained in this small series of cases.

Reed and Denis (1927) consider that inorganic sulphate is present only in blood-serum, none being contained in the corpuscles. In this investigation the blood-serum has been examined in cases of eclampsia and other toxæmias of pregnancy. The method used was described by Cuthbertson and Tompsett (1931), the normal values varying between 0·1 and 0·5 mgm. S per 100 c.c. serum. Tables of results are appended.

In the cases of eclampsia blood-pressure estimations and Esbach readings corresponding to the days on which the tests were made showed wide variations. The highest systolic blood-pressure recorded was 214 mm. Hg. and the lowest 120 mm. Hg., while the Esbach level ranged between 34 and $\frac{1}{2}$ gm. per 1000 c.c. urine.

Previous investigations into the sulphur content of the serum or plasma

TABLE I.—*Inorganic Sulphur and Non-protein-nitrogen in Control Cases.*

Case No.	Age.	Gravida.	Non-protein nitrogen mgm. per 100 c.c. serum.	Inorganic sulphur mgm. S per 100 c.c. serum.	Remarks.
1	26	4	..	0·6	Hyperemesis.
2	38	1	25	0·6	,,
3	22	4	..	0·6	,,
4	22	5	..	0·3	,,
5	28	5	..	0·3	Lactosuria.
6	34	4	25	0·5	Pyelitis.
7	29	1	31	1·3	Normal pregnancy. In labour.
8	20	1	22	1·5	,, ,, ,,
9	20	1	18	1·4	,, ,, ,,
10	23	1	25	1·3	,, ,, ,,
11	19	1	26	1·2	,, ,, ,,
12	30	7	22	1·2	,, ,, ,,
13	24	2	18	1·2	,, ,, ,,
14	38	2	26	1·0	Pyelitis. In labour.
15	26	1	18	0·9	Normal pregnancy. After delivery.
16	21	1	22	1·1	,, ,, ,,
17	27	2	28	1·4	,, ,, ,,
18	38	10	32	1·3	,, ,, ,,
19	18	1	27	1·3	,, ,, ,,
20	29	2	24	1·2	Ante-partum hæmorrhage.
21	26	1	32	1·8	Pre-eclampsia. Esbach 2½. B.P. 145/100.
22	21	1	28	1·8	Pre-eclampsia. Esbach 2½. B.P. 130/85.
23	21	1	23	1·8	Twin pregnancy. Trace of albumen. Œdema. B.P. 130/85.
24	30	1	26	0·5	Nephritic toxæmia. B.P. 148/100.

TABLE II.—*Inorganic Sulphur and Non-protein-nitrogen in Eclampsia.*

Case No.	Age.	Gravida.	Non-protein nitrogen mgm. per 100 c.c. serum.	Inorganic sulphur mgm. S per 100 c.c. serum.	Remarks.
1	41	5	23	2·2	Ante- and intra-partum. 7 fits.
2	19	1	24	2·0	Post-partum. 1 fit. Twin pregnancy.
3	22	1	30	2·6	Intra-partum. 5 fits. Post-partum, 1 fit.
4	32	2	60	2·9	Intra-partum. 1 fit. Previous eclampsia. Death.
5	19	2	30	2·3	Post-partum. 6 fits. Twin pregnancy.
6	29	3	37	2·8	Ante- and intra-partum. 5 fits.
7	17	1	25	2·8	,, ,, 2 fits.
8	30	5	18	2·6	Ante-partum. 5 fits.
9	19	2	48	2·7	,, 6 fits.
10	36	3	46	2·5	,, ,, Death.
11	19	1	30	2·3	Post-partum. 1 fit.
12	19	1	18	2·1	Ante-partum. 2 fits.
13	22	1	38	2·5	Ante- and intra-partum. 12 fits. Diabetic.
14	22	1	30	2·1	Ante-partum and intra-partum. 4 fits.
15	21	1	28	1·1	In labour, before onset of fits.
			32	3·6	Intra-partum. 2 fits. Post-partum, 12. 6th day puerperium.
16	39	5	50	3·6	Ante-partum. 2 fits.
			32	3·2	Fourth day of puerperium.

in disease have been particularly directed towards cases of nephritis with nitrogen retention. It has been shown by Loeb and Benedict (1927), Reed and Denis (1927) and Cuthbertson and Tompsett (1931) that, in cases of nephritis and cardio-renal disease, there is an increased retention of inorganic sulphate coincident with the increased retention of non-protein nitrogen, inorganic sulphate being retained in proportionately greater amount than nitrogen, and this particularly in the lower grades of nitrogen retention.

SUMMARY.

Although little variation in the non-protein nitrogen was noted in the cases of eclampsia studied, the inorganic sulphate content was raised very considerably above the normal. This marked elevation was not observed in normal gestation or in toxæmias of pregnancy other than eclampsia, though in these cases values two or three times the normal were found. It is difficult to appreciate why the inorganic sulphate content of the plasma or serum should be raised in normal pregnancy, unless it be assumed that, relative to nitrogen, inorganic sulphate is a more difficult substance to excrete, and that, therefore, its retention may be an earlier indication of renal impairment than is afforded by the study of the non-protein nitrogen or urea content.

This investigation was carried out during the tenure by one of us (D. F. A.) of a McCunn Research Scholarship. To the McCunn Trustees we would express our sincere thanks.

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REFERENCES.

Cuthbertson, D. P., and Tompsett, S. L.—(1931) *Biochem. J.*, **25**, 1237.
Loeb, R. F., and Benedict, E. M.—(1927) *J. Clin. Invest.*, **4**, 33.
Reed, L., and Denis, W.—(1927) *J. Biol. Chem.*, **73**, 623.

ON THE EFFECT OF INTRAVENOUS INJECTION OF GLYCINE ON THE SERUM CALCIUM.

NOAH MORRIS, J. BASIL RENNIE* and SAMUEL MORRIS.

From the Biochemical Laboratory, University Department of Pædiatrics, Royal Hospital for Sick Children, Glasgow; and the Hannah Dairy Research Institute, Ayr.

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It has long been known that both in tetania parathyreopriva and that due to guanidine poisoning there occurs an increased catabolism of nitrogen (Berkely and Beebe, 1909, Burns, 1917). After parathyroidectomy Justschenko (1913) found an increase in the output of amino-acids, and Burns (1917) reported the same finding after the injection of guanidine. Morris, Watson and Morris (1931) confirmed these findings both for parathyroidectomy

* Carnegie Research Scholar.

and guanidine poisoning. Their results seemed to indicate that there was some relationship between the excess amino-acid nitrogen of the urine and the decrease in the amount of calcium retained. The following series of experiments were undertaken to determine whether amino-acid injected directly into the blood-stream had any effect on the serum calcium.

Glycine was the amino-acid used : 2·5 c.c. of a 20 per cent. solution per kilo body-weight were injected intravenously. In two instances the glycine solution was administered intraperitoneally. Blood was withdrawn for analysis before the injection and once or oftener thereafter. Rabbits and goats were the animals used, the latter so that changes in blood volume, produced by withdrawal of blood, should be kept at a minimum.

TABLE I.—*Effect of Glycine Injection on Serum Calcium and Non-Protein Nitrogen of Whole Blood in Rabbits.*

Rabbit No.	Serum calcium (mgm. per 100 c.c.)	Non-protein nitrogen of blood (mgm. per 100 c.c.)	
1	7·9	42·7	
	6·7	100·0	. 10 mins. after intravenous injection.
2	12·2	40·3	
	9·0	59·9	. 1 hour after 1st ,, ,,
	7·3	56·2	. 1 ,, 2nd ,, ,, (2 hours after 1st injection.)
3	11·2	..	
	8·1	..	. ½ hour after intravenous injection.
4	10·5	29·4	
	9·5	49·1	. ½ ,, ,, ,,
5	9·7	20·0	
	10·1	50·0	. 1 ,, 1st intraperitoneal injection.
	10·3	66·3	. 1 ,, 2nd ,, ,, (3 hours after 1st injection.)
6	9·3	34·2	
	9·8	46·9	. 1 hour after intraperitoneal injection.
	8·7	48·7	. 2 hours ,, intravenous ,, (4 hours after intraperitoneal injection.)
A	11·3	40·1	
	12·2	33·0	. ½ hour after intravenous injection of saline.

In no instance was there noted any manifestation of increased neuromuscular excitability or any other abnormality. The intravenous administration of glycine led to a fall in serum-calcium of 1·0 to 3·1 mgm. per 100 c.c. in the rabbit (Table I), and of 2·4 to 2·9 mgm. per 100 c.c. in the goat (Table II). Two hours after the injection the serum calcium had not yet reached its normal value. The non-protein nitrogen was increased more markedly in the rabbits than in the goats, but in only one rabbit (No. 1) when the blood was withdrawn within 10 minutes of the glycine injection did the increase account for even 50 per cent. of the amount of glycine nitrogen injected. In the goats both the serum protein and the chlorine content of the whole blood were slightly diminished, while the inorganic phosphorus of the serum was very slightly increased. When the glycine solution was injected intra-peritoneally in rabbits the calcium content of the serum was practically unchanged.

TABLE II.—*Effect of Glycine Injection on Composition of Blood in Goats.*

Goat No.		Before.	½ hour after injection.	1 hour after injection.	2 hours after injection.
1	Serum calcium	11·3	8·6	8·8	10·4
	,, inorg. P.	3·5	3·8	3·8	3·6
	,, protein	8·68	8·49	8·58	8·56
	Blood N.P.N.	28·5	35·2	32·9	29·9
	,, Cl	315	308	309	313
2	Serum calcium	10·7	8·3	9·0	10·0
	,, inorg. P.	3·5	3·8	3·7	3·6
	,, protein	8·52	8·40	8·45	8·51
	Blood N.P.N.	31·0	36·1	36·0	33·5
	,, Cl	324	310	312	320
3	Serum calcium	10·9	8·0	8·7	9·3
	,, inorg. P.	3·6	3·9	3·7	3·7
	,, protein	9·05	8·56	8·82	9·0
	Blood N.P.N.	31·1	36·1	32·0	32·0
	,, Cl	313	303	304	312

Results in mgm. per 100 c.c. except protein, where figures represent gm. per 100 c.c.

From these results it is clear that the injection of glycine directly into the blood-stream leads to a fall in serum-calcium. From the non-protein-nitrogen values it is evident that comparatively little of the amino-acid nitrogen remains in the blood. It seems feasible, therefore, that the glycine carried calcium with it into the tissue-cells. The tendency of the serum calcium to return to its normal value is probably due to the deaminization of the glycine with consequent release of the calcium. When the glycine is slowly absorbed, as happens when the injection is into the peritoneal cavity, it will be deaminized as quickly as it is absorbed, with the result that serum calcium is not affected. Greenwald (1925) has suggested that in tetania parathyreopriva calcium is precipitated in the tissues as calcium phosphate. In view of our results it seems that excess production of amino-acids also leads to a withdrawal of calcium from the serum into the tissues.

SUMMARY.

Intravenous injection of glycine solution leads in rabbits and goats to a diminution in serum calcium. It is suggested that excess amino-acid production after parathyroidectomy plays a part in lowering the serum calcium.

We are indebted to Drs. A. McLeod Watson and Jordan for performing the injections on rabbits and goats respectively. It is a pleasure to acknowledge the advice and helpful criticism of Prof. G. B. Fleming and Dr. Norman Wright. Thanks are due to the Medical Research Council for defraying a portion of the expenses of the investigation and for a personal grant to one of us (N. M.).

REFERENCES.

BERKELY, W. N., AND BEEBE, S. P.—(1909) *J. Med. Res.*, **20**, 149.
BURNS, D.—(1917) *Quart. J. Exp. Physiol.*, **10**, 361.
GREENWALD, I., AND GROSS, J.—(1925) *J. Biol. Chem.*, **66**, 217.
JUSTSCHENKO, A. S.—(1913) *Biochem. Z.*, **48**, 61.
MORRIS, S., WATSON, A., AND MORRIS, N.—(1931) *Biochem. J.*, 25, 786.

AN ATTEMPT TO DEMONSTRATE RESIDUAL VIRUS IN MONKEYS WHICH HAD RECOVERED FROM YELLOW FEVER.

E. HINDLE,

The Beit Research Fellow in Tropical Medicine.

From the Wellcome Bureau of Scientific Research, London.

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It is generally agreed that recovery from yellow fever is followed by a high degree of immunity, which may persist without any appreciable diminution throughout the life of the individual. Moreover, the immune bodies generally remain present in the serum in sufficiently high concentration to confer passive immunity on susceptible animals inoculated with reasonable amounts of serum (Hindle, 1930, *a*).

The persistence of yellow fever immunity, as indicated by the power of sera to protect monkeys, has recently been tested by Sawyer (1931) in 60 persons who were supposed to have had the disease 30 to 78 years previously. Five out of 6 sera from persons who had had yellow fever 75 years previously, including one 78 years after the attack, protected *rhesus* monkeys against a large dose of virus. Notwithstanding these cases, there was evidence suggesting that sometimes the concentration of antibodies may gradually diminish until they are no longer demonstrable by ordinary protection tests.

It is well known that in some diseases, *e.g.* many protozoal infections, fowlpox, syphilis, rat-bite fever (*Spirillum minus*), etc., the persistence of a high degree of immunity is linked up with a residual infection, or carrier condition, in which the infective agent remains in the body, and is assumed in some way to reinforce the immune substances and make the subject resistant against reinfection. It seemed of interest, therefore, to see whether any evidence of the persistence of virus could be found in monkeys that had recovered from yellow fever, for if latent infections should exist, they would afford a possible explanation of the recurrence of epidemics in regions that have been apparently free from the disease for many years.

GENERAL COURSE OF THE INFECTION.

When a monkey is inoculated with yellow fever virus, the infective material invades the blood circulation within a few hours and may be recovered from the blood throughout the incubation period, which, in monkeys, is generally from 2 to 5 days. After the appearance of febrile symptoms the infectivity of the blood begins to diminish, and usually disappears before the third or

fourth day of fever. Monkeys generally succumb to the infection within 2 or 3 days of the onset of fever, consequently their blood is still infective at death, but in the case of human beings, death rarely occurs before the fifth or sixth day of fever, often later, and then the blood is no longer infective. As I have suggested previously (Hindle, 1930, *b*), this diminution of infectivity is not necessarily the result of the disappearance of virus, which may still be present in the blood, but is due rather to the development of antibodies, which mask its presence. In support of this view, it has been found that when the febrile attack in monkeys persists longer than usual, after the third or fourth day the blood may produce infection when minimal doses are used, but if larger quantities are injected no infection results (Hudson and Philip, 1929). In the case of man, it is generally assumed that the infectivity of the blood disappears soon after the onset of fever, but this is subject to considerable variation, for in at least two cases in this country, the whole blood was still infective on the fourth day of fever (Hindle, 1930, *b*).

EXPERIMENTS ON THE INFECTIVITY OF TISSUES AT DEATH.

Inoculation Experiments.

Inoculation experiments with the tissues of persons who have succumbed to the disease have generally given negative results, but the presence of antibodies in the blood might explain failures to reproduce the infection by the inoculation of post-mortem material.

In the case of monkeys that have recovered from yellow fever, antibodies seem to be constantly present in the blood, but experiments were made with various tissues that had been washed as thoroughly as possible, in order to remove all serum.

Experiment 1.—Rhesus monkey No. 32 was inoculated with dried virus, attenuated by exposure to air for about 4 weeks, and showed a typical rise in temperature after 5 days' incubation. Fifty-six days after inoculation this animal was bled to death, and its organs perfused with Ringer's solution to remove as much blood as possible. Its tissues were inoculated into 3 monkeys, with the following results :

No. 76.—Inoculated subcutaneously with 1 gm. of the cerebral hemispheres showed no reaction, and when inoculated 50 days later with 0·1 gm. of infected liver material, died of yellow fever after 7 days.

No. 77.—Inoculated subcutaneously with 1 gm. of the washed liver of No. 32, showed no febrile reaction during 10 days. Thirty-eight days later it was inoculated with 1·0 gm. of infected liver and showed a slight rise in temperature (2° F.) on the second day, but no other signs of infection. Nine days after this second inoculation it was again injected with a large dose of virus with negative results.

No. 79.—Inoculated subcutaneously with 1 gm. kidney of No. 32, showed no reaction, and when inoculated 43 days later with 0·1 gm. of infected liver, died of typical yellow fever in 4 days.

The results of this experiment indicate that although kidney and brain-tissue of a recovered monkey produce neither infection nor persistent

immunity in susceptible animals, injection of the liver protected the monkey against subsequent doses of virus.

Experiment 2.—*Rhesus* monkey No. 105 was inoculated with 0·001 gm. of infected liver, and after an incubation period of 3 days had a febrile attack lasting 5 days. It was killed on the ninth day after inoculation and pieces of liver, spleen and kidney carefully washed free from blood. These tissues were then inoculated into three monkeys as follows:

No. 112.—Inoculated subcutaneously with 1 gm. of washed liver material showed a rise in temperature the following day, which gradually subsided. Seventeen days later it was inoculated with infected liver, and after an incubation period of 3 days had a febrile attack also of 3 days and then recovered.

No. 113.—Inoculated subcutaneously with 1 gm. of washed spleen of No. 105, showed no significant rise in temperature and 17 days later was inoculated with infected liver. It reacted in a similar manner to No. 112, showing a febrile attack of 2 days, after which it recovered.

No. 114.—Inoculated subcutaneously with 1 gm. of washed kidney of No. 105, showed a slight rise in temperature on the fourth day, but when inoculated 17 days later with the same dose of infected liver as the two previous monkeys, it died of yellow fever after 5 days.

The results of this experiment also indicate the absence of any infection or signs of protection in the case of the monkey inoculated with kidney tissue. Both the monkeys inoculated with liver and spleen respectively were partially protected against a subsequent dose of virus, since this was a particularly virulent strain, which up to the date in question had killed every monkey inoculated with similar doses of virus.

Experiment 3.—*Rhesus* monkey 117 was inoculated subcutaneously with a mixture of 5 c.c. human convalescent serum and 0·1 gm. of infected liver. No reaction followed, and the animal was subsequently re-inoculated four times with negative results at various intervals with doses of infected liver, ranging up to 3 gm. Four and a half months after the last inoculation it was killed and perfused with Ringer's solution and the liver and spleen washed in 10 per cent. ascitic fluid; then 1·5 gm. of each tissue respectively were inoculated into two normal monkeys, Nos. 208 and 209, neither of which showed any signs of infection. Fifty-eight days later monkey No. 208, inoculated with liver material, received a dose of virus and died of yellow fever after 5 days. The other monkey, inoculated with spleen tissue, was not tested until 4 months after the original inoculation, when it succumbed to a test dose of virus.

The absence of protection in this experiment supports the view that the immunity which may follow the inoculation of the liver or spleen of a recovered animal is a passive one, for if any considerable interval elapses before giving a test dose of virus no immunity is found. This view is supported by the following experiments:

Experiment 4.—Monkey No. YM 20 was inoculated with 0·1 gm. of infected liver but showed no signs of infection, although inoculated twice subsequently with yellow fever material. Five months after the original inoculation it was perfused and 1·0 gm. of the washed liver inoculated into Monkey No. 220.

This animal showed no febrile reaction, and after an interval of 64 days was inoculated with yellow fever virus, and died with typical symptoms of the infection 9 days later.

Experiment 5.—Monkey No. 153 was infected by the bites of mosquitoes but recovered after a mild attack of the disease. It was subsequently reinoculated three times with large doses of virus without any obvious effect. Fourteen days after the last inoculation it was killed, and the liver and kidney washed free from blood and inoculated into two monkeys.

No. 324.—Inoculated intraperitoneally with 4 gm. of washed kidney tissue of No. 153, showed no significant febrile reaction, and when inoculated with virus 34 days later died of yellow fever after 5 days.

No. 325.—Inoculated intraperitoneally with 4 gm. of washed liver of No. 153, remained uninfected, and when inoculated 34 days later with the same dose of virus as No. 324 it also died 5 days later.

Experiment 6.—Kuczynski and Hohenadel (1930) obtained infection with the washed leucocytes from a mild case of yellow fever on the 8th day of the disease, 4 days after fever had disappeared. Accordingly an experiment was made to determine whether the leucocytes of a recovered monkey still contained virus. A normal animal was inoculated intraperitoneally with the leucocytes, washed four times in 10 per cent. ascitic saline solution, from 20 c.c. of the blood of hyper-immune monkey No. 153 (see above, Experiment 5). It showed no reaction during 21 days, and was then reinoculated with similarly washed leucocytes from 30 c.c. of the blood of a second monkey, that after vaccination had been repeatedly inoculated with virus with negative results. No reaction was observed after the second inoculation of leucocytes, and 13 days later the monkey was inoculated with yellow fever virus. The animal showed no signs of infection, although a control monkey inoculated at the same time with a similar dose of the virus died of yellow fever 4 days later.

The results of this experiment suggest that immune bodies are also present in the leucocytes of recovered yellow fever animals, but it is important to note that occasionally *rhesus* monkeys are refractory to infection.

Summarizing the results of the above experiments, it will be seen that out of eleven monkeys inoculated with tissues of recovered animals, washed free from blood, none showed any reactions which could be interpreted as yellow fever. The three monkeys inoculated with kidney tissue, when subsequently inoculated with virus 17, 34 and 43 days respectively after the original injection, all died of typical yellow fever. The monkey inoculated with brain tissue and tested after 50 days also showed no signs of protection. In the case of the animals inoculated with either liver or spleen, there is clear evidence of the production of a passive immunity, for the monkeys inoculated with virus after intervals of 17 days were both immune, as also was one tested after 38 days. On the other hand, two died when tested after an interval of 34 days as well as three others after longer intervals, viz. 58 days, 64 days and 4 months. It has been found that in yellow fever the passive immunity in monkeys inoculated with the immune serum of an homologous species, begins to diminish after 3 weeks and disappears by the end of 7 weeks (Bauer, 1931). The results obtained by the inoculation of the liver and spleen of immune

monkeys agree, therefore, with those obtained by the inoculation of immune serum.

If residual virus had been present in the inoculum a more lasting immunity should have developed, for, as shown previously (Findlay and Hindle, 1931), the simultaneous inoculation of yellow fever virus and immune serum, although producing no obvious signs of infection, is followed by active immunization. It is possible, however, that if only very small amounts of residual virus had been present, the great excess of immune bodies might have prevented the development of any active immunity.

Unfortunately it has not been possible to make exact titration experiments to decide this question, but in one experiment a monkey inoculated with a mixture of 5 c.c. immune serum and 0·1 gm. of infected liver developed an active immunity, as well as a second monkey inoculated with only 1 c.c. of the serum and the same dose of infected liver. In this case an excess of immune serum did not prevent the development of an active immunity, and it seems probable therefore, that had any residual virus been present in the immune monkeys, it would have declared its presence by the production of a more lasting immunity.

FEEDING EXPERIMENTS WITH MOSQUITOES.

The multiplication of virus inside the body of the yellow fever mosquito, *Aëdes ægypti*, suggested the possibility of using this method for detecting the presence of virus in the tissues of recovered animals. Preliminary experiments were made, and no difficulty was encountered in the infection of mosquitoes by feeding them on mixtures of yellow fever virus and honey. After the usual incubation period of 9 to 12 days (at 28° C.) the mosquitoes that had been artificially fed on these mixtures became infective by bite, even when the food material had only been taken into the diverticulum. In this respect the development of yellow fever virus in the mosquito differs from that of *Plasmodium* in *Culex pipiens*, for in this case, as shown by MacGregor (1931), infection of the mosquito only occurs when the blood containing gametocytes is taken into the stomach of the insect, and not if it enters the diverticulum.

Accordingly, experiments were made in which mosquitoes were artificially fed with suspensions of the tissues of immune animals, and then, after a suitable interval, either allowed to bite, or inoculated into, normal monkeys.

The results of three attempts to infect mosquitoes by feeding them with suspensions of the tissues of immune monkeys gave consistently negative results, but there remained the possibility that the development of the virus in the mosquito might be prevented by the simultaneous ingestion of immune bodies. The following experiments were made to decide this question.

Experiment 51.—A batch of mosquitoes was fed on a mixture of hyper-immune serum and yellow fever virus from two infected monkeys, to which was added a little honey immediately before feeding. Fourteen days later the mosquitoes were allowed to bite a normal monkey, which showed no reaction during 10 days, but when inoculated with infected blood, died of typical yellow fever on the 4th day. Forty-two days after the original meal

the surviving mosquitoes were killed and inoculated into a second monkey, which also failed to show any reaction.

Experiment 52.—A batch of mosquitoes was fed on a mixture of infected blood and immune serum, to which was added a little honey. After being kept 14 days at 28° C., these insects were allowed to feed on a normal monkey which failed to show any reaction.

The rather unexpected result of these last two experiments indicates that the presence of a certain quantity of immune serum prevents the yellow fever virus from becoming established in the body of the mosquito. It is only reasonable to assume, therefore, that in the case of mosquitoes fed on suspensions of the tissues of recovered animals, the immune bodies would prevent the development of any possible infection in the mosquito. These experiments indicate that this method of using the intermediate host for the detection of latent virus is not likely to be successful, since the immune bodies seem to prevent the infection of the mosquito as effectively as they do that of the vertebrate host.

CONCLUSIONS.

1. Monkeys inoculated with suspensions of the tissues (brain, liver, kidney and spleen) of animals recovered from yellow fever do not become infected, but those receiving liver and spleen develop a transient immunity. In a single experiment protection was conferred by the inoculation of washed leucocytes from an immune animal.

2. This transient immunity has the duration of that produced by an immune serum alone and is probably passive in nature.

3. Since even a strongly over-neutralized mixture of yellow fever virus and antiserum evokes an active immunity, these findings suggest that virus does not persist in the tissues of recovered monkeys.

4. Mosquitoes fed on mixtures of virus and immune serum do not become infected, and are therefore of no use for demonstrating the possible presence of virus in the tissues of recovered animals.

REFERENCES.

BAUER, J. H.—(1931) *Amer. J. Trop. Med.*, **11**, 451.
FINDLAY, G. M., AND HINDLE, E.—(1931) *Brit. Med. J.*, **1**, 740.
HINDLE, E.—(1930, *a*) *Lancet*, **1**, 451.—(1930, *b*) *Ibid.*, **2**, 835.
HUDSON, N. P., AND PHILIP, C. B.—(1929) *J. Exp. Med.*, **50**, 583.
KUCZYNSKI, M. H., AND HOHENADEL, B.—(1930) *Trans. Roy. Soc. Trop. Med. Hyg.*, **23**, 439.
MACGREGOR, M. E.—(1931) *Ibid.*, **24**, 465.
SAWYER, W. A.—(1931) *J. Prevent. Med.*, **5**, 413.

THE ACTION OF ACETYLCHOLINE ON THE GASTRIC SECRETION IN MAN.

JOHN F. WILKINSON.

From the Department of Clinical Investigations and Research, the University of Manchester and Manchester Royal Infirmary.

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ACETYLCHOLINE has the constitution $(CH_3)_3 \equiv N\,(OH) - CH_2 - CH_2 - O - CO - CH_3$. The free base is extremely hygroscopic and unstable. A relatively stable solution of the hydrochloride or hydrobromide of the base is obtained by the addition of glucose. It has been isolated from fresh animal tissues (Dale and Dudley, 1929), but very readily decomposes into its parent substance, choline, and acetic acid. The physiological effects of acetylcholine were investigated by Dale (1914), Hunt (1914–15, 1918), and Dale and Richards (1918–19), amongst others, who drew attention to the profound depression of the arterial blood-pressure of animals after intravenous injection, primarily due to its dilator action on the arterioles. Acetylcholine, itself, is about one-third as toxic as choline, but 100,000 times more active ; this is offset by the slower absorption of the former from the tissues when given subcutaneously (Wertheimer and Paffrath, 1925). Whereas intravenous injection of the salts of acetylcholine in animals is rapid, its effects when administered subcutaneously or intravenously in man are not so dramatic, and it has been successfully employed in the treatment of certain vascular diseases (Villaret and Justin-Besançon, 1929).

These workers have observed an abundant secretion of pancreatic juice, rich in lipase and amylase, in the dog after administration of acetylcholine. Its stimulating action on the intestines, liver, spleen, submaxillary and lachrymal gland is readily abolished by atropine (Dale, 1914 ; Hunt, 1918 ; Voss, 1926), but judged by its effects on frog's heart, Clark (1926) has demonstrated that this antagonism is one of effects rather than combination.

In the present paper the effects of acetylcholine upon the gastric secretion in man are considered.

The acetylcholine was used in the form of the solid hydrochloride or stabilized in glucose solution (kindly supplied by the Anglo-French Co., Ltd., London), or the hydrobromide stabilized in glucose solution (May and Baker, Ltd., London).

The unstabilized solid was dissolved in the necessary amount of sterile distilled water immediately before use, doses of 0·05–0·2 gm. being employed.

NORMAL SUBJECTS.

The subjects used for this portion of the investigation were either normal individuals, or patients suffering from such complaints as did not show any gastric impairment.

All these selected subjects were first given ordinary gruel test-meals by the fractional method—these being repeated if bile was present in any sample. Subjects showing abnormal forms of gastric secretion were rejected from the series. Several days later the tests were repeated and the acetylcholine injected subcutaneously. The time of injection varied in different tests, but, after removal of fasting contents, was usually given just prior to or $\frac{1}{4}$–$\frac{1}{2}$ hour following the administration of the gruel meal.

Samples (10 c.c.) of the gastric contents were withdrawn at 15-minute intervals, and examined in the usual manner for free and total acidities and total and mineral chlorides.

The results obtained with fourteen subjects fall very clearly into the two groups as follows :

Group I. Showing Responses to Acetylcholine.

This group of 10 cases is illustrated by subjects N. C—, H. E—, J. H— and J. S—.

In subject N. C— a series of fractional gastric analyses were carried out over a period of several weeks, several days being allowed between each consecutive test in order to compare the effects of different doses of acetylcholine. Acetylcholine hydrochloride was used and dissolved in sterile water immediately before use. In Table I are shown separately the titre

TABLE I.—*Subject N. C—, June 8th, 1929, to July 5th, 1929. Free Hydrochloric Acid Secretion after Acetylcholine (in gm. Cl per 100 c.c.).*

Test sample.	Control test.	After subcutaneous injection of—		
		0·1 gm.	0·15 gm.	0·2 gm.
1	0·155	0·106	0·108	0·156
2	0·014	0·000	0·021	0·011
3	0·028	0·032	0·021	0·036
4	0·057	0·061	0·057	0·078
5	0·071	0·103	0·110	0·106
6	0·124	0·135	0·153	0·156
7	0·067	0·138	0·167	0·170
8	0·036	0·135	0·177	0·181
9	..	0·096	0·106	0·114
	Total Chlorides Secretion after Acetylcholine.			
1	0·355	0·355	0·259	0·227
2	0·085	0·053	0·064	0·082
3	0·089	0·082	0·046	0·085
4	0·138	0·163	0·085	0·128
5	0·156	0·206	0·156	0·163
6	0·259	0·262	0·199	0·209
7	0·316	0·208	0·238	0·277
8	0·226	0·305	0·294	0·294
9	..	0·309	0·309	0·309

TABLE II.—*Effect of Acetylcholine on Gastric Secretion in Normal Individuals (Group I).*

Case.	Titre.	Normal gruel test.										After acetylcholine (0·125 gm.).									
		½.	¼.	¾.	1.	1¼.	1½.	1¾.	2.	2¼.	2½.	½.	¼.	¾.	1.	1¼.	1½.	1¾.	2.	2¼.	2½.
J. H—	F.A.	3	10	41	48	49	47	45	38	15	..	2	5	24	43	58	62	63	60	50	..
	T.A.	9	17	49	57	60	58	55	52	24	..	8	11	34	53	68	71	75	72	63	..
	T.C.	15	16	51	58	66	65	63	63	55	..	11	12	33	60	73	75	81	87	80	..
J. S—	F.A.	5	18	36	51	53	40	35	29	29	42	12	37	66	65	58	35	14	19	25	16
	T.A.	15	33	49	63	68	54	45	39	41	54	27	55	80	79	78	48	30	33	39	34
	T.C.	23	50	58	83	83	78	76	68	66	70	33	63	93	96	95	95	57	67	65	72
H. E—	F.A.	4	10	22	32	33	32	32	22	23	..	2	9	34	39	45	43	41	43	36	..
	T.A.	14	24	36	44	46	47	45	35	37	..	10	18	45	52	57	53	61	56	50	..
	T.C.	16	23	43	60	66	66	61	53	60	..	16	23	58	68	77	75	86	74	65	..

F.A. = Free acidities; T.A. = total acidities; T.C. = total chlorides. In Tables II and III, figures represent titration units or c.c. of N/10 reagent required per 100 c.c. gastric juice.

TABLE III.—*Effect of Acetylcholine on Gastric Secretion in Disease.*

Case.	Test.	Free acidities.										Total chlorides.									
		½.	½.	¾.	1.	1¼.	1½.	1¾.	2.	2½.	2½.	½.	½.	¾.	1.	1¼.	1½.	1¾.	2.	2½.	2½.
A. B— (pernicious anæmia)	G.	0	0	0	0	0	0	0	0	..	..	10	3	11	26	32	45	56	42	39	..
	A.	0	0	0	0	0	0	0	0	..	..	8	10	8	11	22	35	68	60	50	..
	H.	0	0	0	0	0	0	0	0	..	..	9	10	12	18	33	48	83	76	68	..
W. S— ,, ,,	G.	0	0	0	0	0	0	0	0	..	..	5	5	5	6	9	18	26	26	..	..
	A.	0	0	0	0	0	0	0	0	..	..	11	8	24	35	41	44	48	..	..	..
	H.	0	0	0	0	0	0	0	0	..	..	10	2	3	16	31	33	34	33	..	..
J. P— ,, ,,	G.	0	0	0	0	0	0	0	0	..	..	4	4	13	18	24	30	..	..	..	..
	A.	0	0	0	0	0	0	0	0	..	..	1	3	7	12	21	24	..	..	..	..
	H.	0	0	0	0	0	0	0	0	..	..	2	5	13	19	39	30	36	..	..	..
A. W— (mucous colitis)	G.	0	0	0	0	0	0	0	0	..	..	12	12	28	50	55	54	51	49	..	..
	A.	0	0	0	0	0	0	0	0	..	..	3	8	14	30	46	57	57	63	..	..
	H.	0	0	1	6	5	5	0	0	..	..	9	9	23	43	55	64	69	83	..	..
R. S— (idiopathic anæmia)	G.	0	0	0	0	7	3	3	0	..	..	15	18	20	22	30	32	31	..	..	..
	A.	0	0	0	14	5	0	0	0	..	..	18	23	28	39	39	28	..	..	..	..
	H.	0	7	32	48	33	6	0	0	..	..	16	37	53	67	61	43	28	..	..	..
B. B— ,, ,,	G.	0	3	4	11	16	15	15	9	4	5	16	16	12	22	39	48	50	47	52	53
	A.	3	6	16	24	36	41	30	24	23	16	15	16	36	48	67	74	69	70	72	62
A. N. W— (duodenal ulcer)	G.	0	16	35	61	77	80	90	99	102	100	20	25	56	83	95	101	113	121	126	123
	A.	21	36	54	65	78	85	99	101	104	109	43	52	63	77	95	107	118	120	128	123
C. H— (gastric ulcer)	G.	9	9	32	51	54	59	61	62	61	..	16	21	50	77	80	87	90	88	100	..
	A.	5	30	50	63	62	65	63	68	82	..	10	45	63	85	90	90	95	97	110	..
E. W— (Raynaud's disease)	G.	0	1	7	16	27	35	24	11	8	10	21	19	21	37	51	74	72	64	60	72
	A.	0	0	12	18	45	61	68	75	56	..	22	16	18	34	71	91	100	109	105	..
	A.A.	0	0	0	0	0	5	13	16	23	..	10	7	14	16	16	25	39	43	47	..

G. = Gruel meal; A. = after acetylcholine; H. = after histamine; A.A. = after atropine sulphate (0·32 mgm.), followed by acetylcholine (0·1 gm.) 15 minutes later.

values for the free acidities, and total chlorides after subcutaneous injection of 0·1, 0·15 and 0·2 gm. respectively of acetylcholine hydrochloride 15 minutes before the first gruel samples were withdrawn.

It will be observed that in these three tests the main secretory titres increased only slightly with the dosage, so that the maximum effect was obtained with the dose of 0·2 gm. The free and total acidities showed definite increases in titre after acetylcholine, whereas the total chlorides gave less variation, the increased hydrochloric acid secretion being compensated for by a reduction in the mineral chloride secretion.

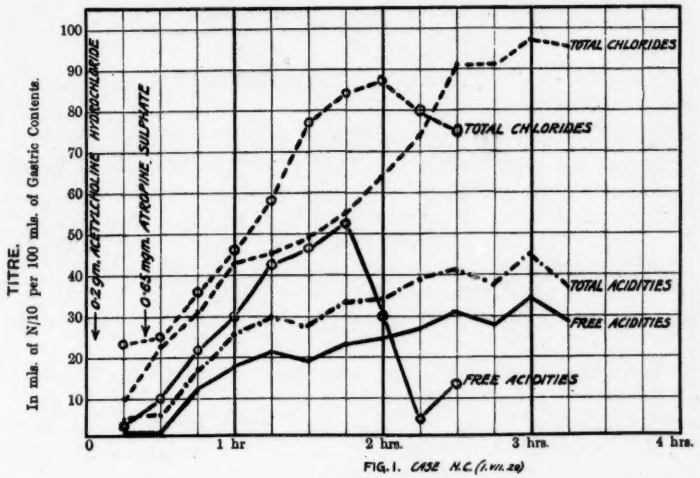

FIG. 1.

AFTER ACETYLCHOLINE (0·2 gm.)

o——o——o Free acidities.
o - - - - - o - - - - - o Total chlorides.

AFTER ACETYLCHOLINE (0·2 gm.) AND ATROPINE SULPHATE (0·65 mgm.)—

——— Free acidities.
—·—·—·—·—·—·— Total acidities.
- - - - - - - - - - - - - - - Total chlorides.

In a further experiment on subject N. C—, acetylcholine hydrochloride (0·2 gm.) was given subcutaneously 15 minutes before withdrawing the first gruel sample, followed by atropine sulphate (0·65 mgm.) 15 minutes later. In Fig. 1 it will be seen that there was a marked depression of the gastric secretory functions (*cf.* Table III, Case E. W—). This antagonizing action of atropine towards acetylcholine has already been noted with regard to other organs and tissues (*vide supra*).

The 10 cases examined in this group showed similar stimulation of the gastric secretion following the use of acetylcholine salts ; thus, in Table II (subjects J. H—, J. S— and H. E—) are shown the results after subcu-

taneous injection of 0·125 gm. of the hydrobromide in glucose solution. It will be seen that in these cases there was a definite increase in the titres for the free and total acidities and total chlorides, but no alteration in the emptying times of the stomachs.

Group II. No Effect on Gastric Secretion.

Four subjects failed to show any alteration in gastric secretions after acetylcholine (0·1 gm. hydrochloride; 0·125 gm. hydrobromide). There were no marked differences between the acidity titres, but a tendency for some of the total chlorine values to show very slight increases. No alterations were noted in the emptying times of the stomach.

ABNORMAL CONDITIONS.

PERNICIOUS ANÆMIA.

From a consideration of the results obtained in other cases and on normal individuals, it was not anticipated that acetylcholine would produce any marked alterations in the gastric condition, especially in view of the fact that histamine had failed to produce free acid in these cases.

A series of 25 cases of pernicious anæmia were examined. In each subject (clinically typical of severe pernicious anæmia) an ordinary fractional gastric analysis was carried out with the usual gruel meal. In several cases this was repeated, until a complete analysis was obtained with all samples free from bile.

Some 3–10 days later the test was repeated, and acetylcholine hydrochloride (0·05–0·15 gm.) stabilized in glucose solution was injected subcutaneously 15 minutes before the first gruel sample was withdrawn. There was little difference in the effects produced by the different doses used.

In the 25 cases examined the achlorhydria and low total acidities persisted. The total and mineral chlorides showed certain alterations, and in every case mucus was much diminished after acetylcholine. There were no alterations in the emptying-times of the stomachs. The results may be considered in three groups:

Group I. Increased Chloride Secretion.

The 12 cases in this group showed slight to marked increase in the secretions of total (and mineral) chlorides after acetylcholine; 10 of these disclosed slight peptic activities in the samples obtained (*cf.* Table III, Cases A. B— and W. S—).

Group II. No Alterations in Chloride Secretion.

Twelve cases did not show any alteration in the chloride (total and mineral) secretion or in the peptic activities.

Group III. Diminished Chloride Secretion.

One case showed a slight fall in total and mineral chlorides after injections of acetylcholine.

In view of the observations of Johanson (1929) and others, 16 of these cases were given histamine acid phosphate (0·25–0·5 mgm.) subcutaneously in subsequent test-meals, and the results corresponded very closely with those obtained after acetylcholine.

Thus, 7 of the subjects were from Group I, and gave increased chloride secretion after histamine as well as acetylcholine (see Table III, Case A. B—), while 7 were from Group II, and showed no alterations in gastric secretions after histamine or acetylcholine.

Of the two remaining, one showed increased chloride secretion after histamine, but not acetylcholine (Table III, Case J. P—), and the other after acetylcholine, but not histamine ; in the latter case histamine showed a reduced chloride secretion.

ACHLORHYDRIA.

In 3 cases of achlorhydria associated with mucous colitis acetylcholine (0·1 gm.) failed to stimulate the production of free hydrochloric acid, although there was an increase in the total and mineral chloride secretion (Table III, Case A. W—).

Comparison with histamine (0·5 mgm.) in the same case shows the production of free acid in three samples. This case has been under my observation for over two years without any alteration in the gastric condition, which has been investigated some 30 times during that period with these substances. Prior to coming under my observation she had given herself gastric lavage daily for 18 months by means of a full-size stomach-tube.

Thus, it will be seen that pseudo-achlorhydria is not disclosed by acetylcholine, which cannot therefore replace histamine for the detection of such cases.

Exactly comparable results were obtained in two cases of apparent achlorhydria associated with malignant disease of the stomach.

HYPO-ACIDITY.

A series of cases showing marked hypo-acidity associated with chronic idiopathic anæmia of the secondary type and no demonstrable organic lesions were examined in a similar manner.

After acetylcholine (0·125 gm. hydrobromide ; 0·1 gm. hydrochloride) there was an increased secretion of free acid and total chlorides, but to a lesser degree than was obtained after histamine (*cf.* Table III, Cases R. S— and B. B—).

HYPERACIDITY.

Cases of gastric and duodenal ulcers, associated with hyperacidity, showed varying responses after acetylcholine from no change to moderate increase in free acid and total chloride secretion (*cf.* Table III, Cases A. N. W—, C. H—).

RAYNAUD'S DISEASE.

A woman with Raynaud's disease was the subject of some 20 different experiments, and always passed the tube herself without difficulty. From time to time ordinary control fractional test-meals were given, and all showed a maintenance of the normal type of curve for this case (Table III, Case E. W—).

At intervals of several days various experiments were carried out (Table III, Case E. W—), and it was found that :

(1) There was a markedly increased secretion of free acid and total chlorides after 0·05–0·15 gm. of acetylcholine hydrochloride.

(2) Atropine (0·32 mgm.) prevented very considerably the stimulating effect of acetylcholine (0·1 gm.).

CARDIOSPASM.

A case of cardiospasm with clinical features similar to the previous case was examined and yielded results almost identical with those obtained above.

SUMMARY.

1. The stimulating influence of acetylcholine on gastric secretion has been investigated in normal and abnormal subjects.

2. Its effects on the gastric secretion in normal and abnormal individuals are uncertain.

3. In the majority of normal cases there is an increased secretion of free hydrochloric acid and total chlorides following injection, but it is much less marked than that produced by histamine.

4. The response increases slightly with the dose of acetylcholine used.

5. A small group of normal subjects failed to display any alteration in secretion.

6. Acetylcholine is not so powerful as histamine, and frequently fails to disclose pseudo-achlorhydric conditions.

7. It has rather more marked effects in cases showing hypo-acidity and Raynaud's disease, but here also histamine is more powerful.

8. Atropine readily counteracts or prevents the effects of acetylcholine on the gastric secretory mechanism.

REFERENCES.

CLARK, A. J.—(1926) *J. Physiol.*, **61**, 547.
DALE, H. H.—(1914) *J. Pharmacol.*, **6**, 147.
DALE, H. H., and DUDLEY, H. W.—(1929) *J. Physiol.*, **68**, 97.
DALE, H. H., AND RICHARDS, A. N.—(1918–19) *Ibid.*, **52**, 110.
HUNT, R.—(1914–15) *J. Pharmacol.*, **6**, 477 ; (1918) *Amer. J. Physiol.*, **45**, 197, 231.
JOHANSEN, A. H.—(1929) *J. Amer. Med. Ass.*, **92**, 1728.
VILLARET, M., AND JUSTIN-BESANÇON, L.—(1929) *Lancet*, **1**, 493.
VOSS, O.—(1926) *Arch. exp. Path. Pharmak.*, **116**, 367.
WERTHEIMER, E., AND PAFFRATH, H.—(1925) *Arch. ges. Physiol.*, **207**, 254.

THE BASAL OXYGEN CONSUMPTION OF GOITROUS AND NON-GOITROUS RATS.*

A. W. SPENCE.

From the Department of Physiology, St. Bartholomew's Hospital, London.

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IT has been shown in a previous paper (Spence, 1932) that feeding rats on a diet deficient in vitamins A, B, C and D produces simple goitre in a large percentage of animals. With one exception, every gland presented evidence of exhaustion, resulting in the formation from the vesicular epithelium of keratinization cysts. In this connection it was therefore of interest to determine the basal oxygen consumption of the animals at intervals during the period that they were under experimental conditions. This was done every two to three months, both on the 24 rats fed on the vitamin-deficient diet, and on the 6 normal controls of the same age.

EXPERIMENTAL.

Method.—The determinations were carried out under standard conditions, which were procured by withholding all food from the animal for 16 to 22 hours before the estimation. Further, Benedict and Macleod (1928–29, *b*) have shown that the environmental temperature in which the rats live has a distinct effect on the metabolism; the heat production decreases with increasing environmental temperature. In the present experiments the temperature of the room in which the animals were housed was kept reasonably steady (22·5° to 29·5° C.), so that variations in the environmental temperature cannot be regarded as a complicating factor.

By determining the oxygen consumption of the rats under basal conditions a measure of the basal metabolism was obtained. The apparatus used was that devised by Richards and Collison (1928). The writer is indebted to Dr. J. H. Gaddum, through whom the instrument was obtained from the National Institute for Medical Research, Hampstead. The method permits the graphic registration of the oxygen consumption and of the movements of the animal. The animal is placed in a closed chamber, which is immersed in a water-bath maintained at 25° C., and the carbon dioxide and water produced are absorbed by soda-lime. Oxygen bubbles in through a valve containing liquid paraffin to keep the pressure constant in the chamber, and the entrance of each bubble is recorded on a slowly revolving drum. The oxygen consumption is determined from the rate of formation of the bubbles. Any movement of the animal produces a distortion of the record and irregularities in the rate of formation of the bubbles. The method thus has the great advantage that it is possible to tell without difficulty the parts of the record which correspond to periods during which the animal is at rest.

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The fasting rat is placed in the chamber for 2½ hours. A tracing is not made during the first hour, to allow the temperature of the chamber to reach the temperature of the thermostat, and to allow the animal to get used to the conditions. Then a tracing is made until the end of the experiment, and the bubbles are counted in all the periods during which the animal is at rest, neglecting those immediately before and after movement, when their rate of formation is obviously increased. The barometric pressure is taken during the experiment. The volume of oxygen obtained is reduced to 0° C. and 760 mm. pressure.

Accuracy of the method.—In order to test the accuracy of the method, five determinations were made on two normal male rats, aged 5 months, over a period of 11 days. The results are expressed in cubic centimetres of oxygen at 0° C. and 760 mm. pressure consumed per square metre of body surface per minute. The surface area was calculated from Meeh's formula (1879), in which—

$$A = kW^{\frac{2}{3}},$$

where A = area in square centimetres, W = weight in grammes, and k = constant. The constant originally applied by Rubner for albino rats was 9·1. A. V. and A. M. Hill (1913) concluded from actual measurements of the surface area that the constant was 10, while Giaja (1925) proposed the value 8·99. Carman and Mitchell (1926) have recently suggested the value 11·36, but, as Benedict and MacLeod (1928–29, *a*) have pointed out, this is too high, because they stretched the skins of their rats and the area measured was therefore too large. Benedict and MacLeod (1928–29, *a*) concluded from a survey of the literature that the most accurate constant was that determined by Rubner, *i. e.* 9·1. The value of the constant used in the present calculations of the surface area, therefore, was 9·1.

TABLE I.—*Showing the Basal Oxygen Consumption of 2 Normal Male Rats, aged 5 months, over a period of 11 days. (Temperature of metabolic chamber at 25° C. Volumes expressed at 0° C. and 760 mm. pressure.)*

Rat.	Day.	C.c. oxygen per minute.	Weight in gms.	Surface in sq. cms.	C.c. O_2 per minute per sq. metre.	Remarks.
A	1	2·854	107	205·0	139·2	Average basal oxygen consumption = 136·3 c.c. per sq. metre per min. Average error = 4·9 per cent.
	3	2·656	107	205·0	129·5	
	5	2·911	103	199·9	145·6*	
	8	2·472	100	196·0	126·1	
	11	2·800	102	198·6	141·0	
B	1	2·894	101	197·3	146·7	Average basal oxygen consumption = 138·7 c.c. per sq. metre per min. Average error = 3·2 per cent.
	3	2·607	99	194·7	133·9*	
	5	2·494	93	186·8	133·5	
	8	2·685	95	189·4	141·7	
	11	2·660	98	193·4	137·6	

* High figure due to discomfort of the animal due to slight leakage of soda lime.

The data of the five determinations on these two rats are charted in Table I. Considering the nature of the estimations, the degree of variation in the different results was not great. The average oxygen consumption in one rat

(A) was 136·3, and in the other (B) 138·7 c.c. at 0° C. and 760 mm. per square metre per minute. The average percentage error was 4·9 and 3·2 respectively. (The higher percentage error in the case of rat A was probably because during the third determination a few particles of soda lime fell into the animal's eyes, causing it discomfort, and thus raising the figure obtained for the oxygen consumption.)

The Basal Oxygen Consumption of Normal Rats.

Determinations of the basal oxygen consumption of the 6 control rats were made every 2 to 3 months over a period of 8 months in one animal, and of 12 months in the remaining 5. When the initial determinations were made, the ages of the rats varied from 50 to 63 days. The results are shown in Table II, and a composite curve in Fig. 1, in which are plotted the average results obtained.

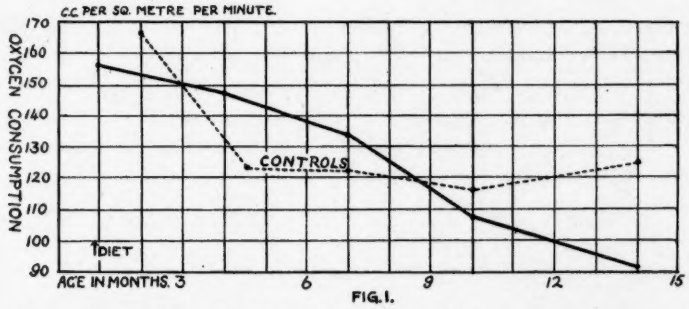

FIG. 1.—Composite curves showing the trend of the average basal oxygen consumption of six normal rats (interrupted line) from the age of 2 to 14 months, and of four rats of the same age fed on a vitamin-deficient diet for periods varying from 10 to 13 months.

High values for the oxygen consumption were obtained in all the animals when they were young and gaining rapidly in weight. When they reached the age of 4½ months and their rate of growth had diminished the values fell considerably, and remained at a fairly steady level during the rest of their lives.

How the basal metabolism in rats should be expressed in order to make the results properly comparable has been disputed. Mørch (1929) has shown in mice that when the animal is gaining in weight, the carbon dioxide production increases proportionately to the surface of the animal, and considers that when such is the case the carbon dioxide production per square centimetre of body surface should be used as the basis for the calculation of the increase. It was found, however, by A. V. and A. M. Hill (1913) that the metabolism of adult rats was more nearly proportional to their weight than to their surface. In the present experiments the results obtained were more in confirmation of Mørch's findings than of those of the Hills. Table III shows that in the writer's rats, which were gaining in weight, the basal oxygen consumption

was more nearly proportional to their surface area than to their weight. For this reason all the results have been expressed in cubic centimetres of oxygen consumed per square metre of surface per minute.

TABLE II.—*Showing the Basal Oxygen Consumption of* 6 *Normal Rats, Initial Age being* 50 *to* 63 *days, over a period varying from* 8 *to* 12 *months. (Temperature of metabolism chamber at* 25° *C. Volumes expressed at* 0° *C. and* 760 *mm. pressure.)*

Rat.	Sex.	Date.	Age in months.	Weight in gms.	C.c. oxygen per minute.	Surface in sq. cms.	C.c. O_2 per sq. metre per minute.
76	F.	31.i.30	2	99	3·298	194·7	169·4
		10.iv.30	4½	165	3·335	273·7	121·9
		27.vi.30	7	170	3·203	279·2	114·9
		1.x.30	10	181	3·345	291·1	114·9
		2.ii.31	14	184	3·598	294·4	122·2
77	F.	31.i.30	2	83	3·298	187·0	176·4
		10.iv.30	4½	157	3·170	264·8	119·7
		27.vi.30	7	167	3·261	276·0	118·2
		2.x.30	10	180	3·335	290·1	115·1
		3.ii.31	14	199	3·852	310·1	124·2
78	F.	4.ii.30	2	80	2·812	168·9	166·4
		10.iv.30	4½	168	3·262	277·0	117·8
		28.vi.30	7	181	3·656	291·2	125·6
		2.x.30	10	181	3·345	291·2	114·9
		4.ii.31	14	189	3·642	292·9	121·5
79	M.	4.ii.30	2	119	3·317	220·2	150·7
		11.iv.30	4½	214	3·739	325·5	114·9
		30.vi.30	7	231	4·250	342·5	124·1
		3.x.30	10	238	4·068	349·5	116·4
80	F.	13.ii.30	2½	90	2·794	182·7	152·9
		11.iv.30	4½	141	2·950	246·5	119·7
		30.vi.30	7	160	3·070	268·2	114·5
		3.x.30	10	170	3·203	279·2	114·9
		5.ii.31	14	187	3·620	297·5	121·7
81	M.	13.ii.30	2½	134	4·343	238·3	182·2
		11.iv.30	4½	218	4·745	329·6	144·0
		30.vi.30	7	246	4·820	357·2	134·9
		4.x.30	10	278	4·728	387·5	122·0
		5.ii.31	14	287	5·394	395·9	136·2

The Basal Oxygen Consumption of the Rats Fed on a Vitamin-Deficient Diet.

In the case of the 24 animals on the vitamin-deficient diet, initial determinations of the oxygen consumption under basal conditions were made immediately the diet was begun, and were repeated every 3 months. The results obtained showed variations in different animals, the reason probably being that occasional rises in the oxygen consumption were brought about by

the secondary infections, from which so many of the animals were suffering. Seven animals died within 6 months, so that in them only one or two determinations were made. Of the remainder the majority showed an initial high oxygen consumption of a similar order to the controls, but the decrease in

TABLE III.—*Showing the Basal Oxygen Consumption to be more nearly proportional to Surface Area than to Weight.*

Rat.	A. Weight ratio.	B. Surface ratio.	C. Oxygen consumption ratio.	Difference between A. and C.	Difference between B. and C.
76	$\frac{184^{(a)}}{99} = 1{\cdot}86$	$\frac{294{\cdot}4^{(b)}}{194{\cdot}7} = 1{\cdot}51$	$\frac{3{\cdot}598^{(c)}}{3{\cdot}298} = 1{\cdot}09$	0·77	0·42
	$\frac{184^{(d)}}{165} = 1{\cdot}115$	$\frac{294{\cdot}4^{(e)}}{273{\cdot}7} = 1{\cdot}075$	$\frac{3{\cdot}598^{(f)}}{3{\cdot}335} = 1{\cdot}075$	0·040	0
77	$\frac{199}{83} = 2{\cdot}40$	$\frac{310{\cdot}1}{187{\cdot}0} = 1{\cdot}66$	$\frac{3{\cdot}852}{3{\cdot}298} = 1{\cdot}17$	1·23	0·49
	$\frac{199}{157} = 1{\cdot}267$	$\frac{310{\cdot}1}{264{\cdot}8} = 1{\cdot}171$	$\frac{3{\cdot}852}{3{\cdot}170} = 1{\cdot}215$	0·052	0·044
78	$\frac{189}{80} = 2{\cdot}36$	$\frac{292{\cdot}9}{168{\cdot}9} = 1{\cdot}77$	$\frac{3{\cdot}642}{2{\cdot}812} = 1{\cdot}30$	1·06	0·47
	$\frac{189}{168} = 1{\cdot}125$	$\frac{292{\cdot}9}{277{\cdot}0} = 1{\cdot}082$	$\frac{3{\cdot}642}{3{\cdot}262} = 1{\cdot}114$	0·011	0·032*
79	$\frac{238}{119} = 2{\cdot}00$	$\frac{349{\cdot}5}{220{\cdot}2} = 1{\cdot}59$	$\frac{4{\cdot}068}{3{\cdot}317} = 1{\cdot}23$	0·77	0·36
	$\frac{238}{214} = 1{\cdot}112$	$\frac{349{\cdot}5}{325{\cdot}5} = 1{\cdot}073$	$\frac{4{\cdot}068}{3{\cdot}739} = 1{\cdot}088$	0·024	0·015
80	$\frac{187}{90} = 2{\cdot}08$	$\frac{297{\cdot}5}{182{\cdot}7} = 1{\cdot}63$	$\frac{3{\cdot}620}{2{\cdot}794} = 1{\cdot}30$	0·78	0·33
	$\frac{187}{141} = 1{\cdot}326$	$\frac{297{\cdot}5}{246{\cdot}5} = 1{\cdot}207$	$\frac{3{\cdot}620}{2{\cdot}950} = 1{\cdot}227$	0·099	0·020
81	$\frac{287}{134} = 2{\cdot}14$	$\frac{395{\cdot}9}{238{\cdot}3} = 1{\cdot}66$	$\frac{5{\cdot}394}{4{\cdot}343} = 1{\cdot}24$	0·90	0·42
	$\frac{287}{218} = 1{\cdot}3165$	$\frac{395{\cdot}9}{329{\cdot}6} = 1{\cdot}201$	$\frac{5{\cdot}394}{4{\cdot}745} = 1{\cdot}137$	0·1795	0·064

$(a) = \frac{\text{Weight on 2.ii.31}}{\text{Weight on 31.i.30}}$. $(b) = \frac{\text{Area on 2.ii.31}}{\text{Area on 31.i.30}}$. $(c) = \frac{\text{O}_2 \text{ consumption on 2.ii.31}}{\text{O}_2 \text{ consumption on 31.i.30}}$.

$(d) = \frac{\text{Weight on 2.ii.31}}{\text{Weight on 10.iv.30}}$. $(e) = \frac{\text{Area on 2.ii.31}}{\text{Area on 10.iv.30}}$. $(f) = \frac{\text{O}_2 \text{ consumption on 2.ii.31}}{\text{O}_2 \text{ consumption on 10.iv.30}}$.

(See Table II.)

* An exception.

oxygen consumption with diminishing rate of growth was more gradual. In several the initial results were unusually low, but rose after three months to about the same level as those of the rest of the animals, and thereafter fell at the same gradual rate. From a study of the figures (Tables IV and V), it will be seen that on the whole the oxygen consumption of the experimental animals

after 3 and 6 months on the diet was at a higher level than that of the controls at the same period. The possibility of this being caused by infection should be considered.

TABLE IV.—*Showing the Variations in Oxygen Consumption which occurred in the Majority of Rats Fed on a Vitamin-Deficient Diet. The results of* 8 *animals taken at random are given. (Temperature of metabolism chamber at* 25° *C. Volumes expressed at* 0° *C. and* 760 *mm. pressure.)*

Rat.	Sex.	Date.	Weight in gms.	C.c. oxygen per minute.	Surface in sq. cms.	C.c. O_2 per sq. metre per minute.
67	M.	21.i.30	42	1·753	109·9	159·5
		4.iv.30	97	3·504	191·9	182·4
		4.vii.30	124	2·694	226·3	119·1
		27.x.30	157	2·998	264·8	113·2
		29.i.31	224	3·814	335·6	113·7
74	F.	28.i.30	38	1·460	102·9	142·0
		8.iv.30	85	2·484	175·9	141·8
		9.vii.30	90	2·145	182·7	117·4
		28.x.30	84	2·015	174·6	115·5
60	M.	14.i.30	52	2·156	126·7	170·1
		1.iv.30	84	2·334	174·6	133·7
		2.vii.30	91	2·257	184·1	122·6
61	F.	15.i.30	30	1·155	87·8	104·4
		2.iv.30	74	2·556	160·4	159·4
		2.vii.30	75	2·198	161·9	135·8
		21.x.30	79	2·000	167·5	119·4
72	M.	28.i.30	50	1·150	123·5	93·1
		8.iv.30	89	2·060	181·4	113·6
		8.vii.30	80	2·470	169·0	146·2
		27.x.30	125	2·368	227·4	104·1
65	M.	20.i.30	40	1·161	106·4	109·0
		3.iv.30	92	2·252	185·4	121·4
		3.vii.30	71	2·084	156·0	133·6
		23.x.30	108	2·746	206·3	133·1
		28.i.31	165	3·410	273·7	124·6
71	F.	25.i.30	43	1·616	111·7	144·7
		5.iv.30	79	2·260	167·5	156·4
		8.vii.30	84	2·092	174·6	119·8
		24.x.30	110	2·523	208·8	120·8
		30.i.31	139	3·502	244·1	143·5
75	F.	29.i.30	51	1·656	125·2	132·3
		9.iv.30	114	2·405	214·0	112·4
		9.vii.30	99	2·411	194·7	123·9
		29.x.30	123	2·341	225·0	104·0
		1.ii.31	144	2·940	250·0	117·6

Thus, in most rats, even in those that were goitrous, there was no significant alteration in the oxygen consumption to suggest a definite thyroid disorder. However, of 11 rats, 4 showed a decrease in oxygen consumption below normal after being on the diet for 9 months, and a further decrease after 13 months (Table V and Fig. 1). These 4 had small goitres, 3 colloid and 1 moderately hyperplastic.

TABLE V.—*Showing the Fall in Oxygen Consumption below normal in 4 Rats Fed on a Vitamin-Deficient Diet. (Temperature of metabolic chamber 25° C. Volumes expressed at 0° C. and 760 mm. pressure.)*

Rat.	Sex.	Date.	Weight in gms.	C.c. oxygen per minute.	Surface in sq. cms.	C.c. O_2 per sq. metre per minute.
57	F.	11·i.30	43	1·979	111·7	177·1
		2.iv.30	90	2·794	182·7	153·0
		1.vii.30	90	2·800	182·7	149·8
		10.x.30	121	2·473	222·5	111·1
		27.i.31	144	2·478	250·0	99·1
73	F.	28.i.30	53	2·035	128·4	158·5
		8.iv.30	132	3·101	235·9	131·5
		9.vii.30	151	3·388	258·0	134·3
		28.x.30	148	2·842	254·7	111·7
		31.i.31	144	2·650	314·7	84·2
52	M.	7.i.30	28	1·354	83·9	161·4
		29.iii.30	97	2·822	192·1	150·4
		1.vii.30	112	2·767	211·4	131·0
		7.x.30	171	3·103	280·3	110·7
58	M.	14.i.30	30	1·147	87·8	130·6
		1.iv.30	86	2·772	177·3	156·4
		2.vii.30	94	2·257	188·1	120·0
		21.x.30	126	2·270	228·7	99·2

DISCUSSION.

The consistent results obtained in repeated determinations on the same animal demonstrate the accuracy of the method employed for measuring the basal oxygen consumption. The observations on the normal control rats afford a basis for comparison with the findings in the goitrous animals.

The study of the basal metabolism in relation to age has been made more in man than in laboratory animals. In newly born infants the metabolism is very low. At the age of about one year the maximum metabolism is attained; thereafter it falls off rather rapidly until the age of about 15 years, and from then on until old age there is a steady, though slight, decrease in metabolism (Benedict and MacLeod, 1928–29, *b*). The results obtained in the normal control rats thus agree fairly closely with the findings in the human being, but are not in accordance with those obtained in rats by Benedict and MacLeod (1928–29, *b*). In their experiments, from the age of 2 months the basal metabolism rose gradually with increasing age. Their estimations, however, were not made on the same rats throughout, but on individual rats at different ages, although not infrequently the same rat was studied at two or more ages. After a study of the basal metabolism of about 100 rats during the last $2\frac{1}{2}$ years, the writer has come to the conclusion that the variations in different rats of the same age may in some cases be considerable.

The complicating factor of infection in the rats fed on a vitamin-deficient diet confuses to a certain extent the interpretation of the findings. Infection was, in all probability, responsible for the maintenance of the high level of oxygen consumption at the end of the third and sixth months, and for subsequent elevations in different animals. There was no reason to believe that these were due to true hyperthyroidism.

It has been observed that 4 rats with simple goitre showed a fall in oxygen consumption below normal after 9 months. At autopsy the glands of 3 were colloid and of 1 moderately hyperplastic, so that the depression of the oxygen consumption bears no necessary relationship to the histological findings at autopsy in so labile a tissue as the thyroid gland. The thyroid enlargement associated with a fall in metabolism would indicate a lowered efficiency of the gland, resulting in a diminished secretion of thyroid hormone. Decrease in basal metabolism has likewise been observed by Webster, Clawsen and Chesney (1928) in rabbits with hyperplastic goitre, and also by other observers (Plummer, 1927 ; DuBois, 1927) in human beings in whom simple goitre has arisen. When the evidence of thyroid exhaustion, indicated by the formation of keratinization cysts, is considered, it is surprising that similar results were not obtained in the majority of the rats. That they were not illustrates that, in spite of the prolonged strain thrown on the thyroid by dietetic insufficiency and infection, it possesses considerable reserve power, and is often capable of meeting the needs of the body over long periods of stress.

SUMMARY.

1. Simple goitre was produced in the majority of 24 young rats fed on a vitamin-deficient diet. Determinations of the basal oxygen consumption were made at 2- to 3-monthly intervals on the 24 experimental animals, and on 6 normal controls of the same age, by means of the apparatus devised by Richards and Collison.

2. A series of determinations on the normal rats over a period of 12 months showed that when the animals were 2 months old and were gaining rapidly in weight their oxygen consumption was high, but when they reached the age of 4½ months and their rate of growth was diminished, their oxygen consumption fell considerably, and maintained a fairly constant level for the rest of their lives.

3. The basal oxygen consumption of normal rats, which were gaining in weight, was more nearly proportional to their body surface than to their weight.

4. The findings in the rats fed on a vitamin-deficient diet were liable to variations due to the prevalence of secondary infections. The majority showed no significant alteration in basal oxygen consumption which would point to a definite thyroid disorder. Four goitrous animals, however, showed a fall in oxygen consumption below normal after 9 months.

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REFERENCES.

Benedict, F. G., and MacLeod, G.—(1928–29, *a*) *J. Nutrition*, **1**, 343.—(1928–29, *b*) *Ibid.*, **1**, 367.

Carman, G. G., and Mitchell, H. H.—(1926) *Amer. J. Physiol.*, **76**, 380.

DuBois, E. F.—(1927) ' Basal Metabolism in Health and Disease,' Philadelphia (Lea & Febiger). 2nd edition, p. 288.

GIAJA, J.—(1925) *Ann. Physiol.*, **1**, 607.
HILL, A. V., AND A. M.—(1913) *J. Physiol.*, **46**, 81.
MEEH, K.—(1879) *Z. Biol.*, **15**, 425.
MØRCH, J. R.—(1929) *J. Physiol.*, **67**, 221.
PLUMMER, H. S.—(1927) 'The Oxford Medicine,' London (Oxford University Press). **3**, p. 852.
RICHARDS, A. N., AND COLLISON, L. W.—(1928) *J. Physiol.*, **66**, 299.
RUBNER, M.—Quoted from Benedict and MacLeod (1928–29, *a*).
SPENCE, A. W.—(1932) *Brit. J. Exp. Path.*, **13**, 157.
WEBSTER, B., CLAWSEN, I. A., AND CHESNEY, A. M.—(1928) *Johns Hopk. Hosp. Bull.*, **43**, 278.

THE EFFECT OF VITAMIN DEFICIENCY ON THE STRUCTURE OF THE THYROID AND THYMUS GLANDS.*

A. W. SPENCE.

From the Department of Physiology, St. Bartholomew's Hospital, London.

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THESE experiments were undertaken with a view to studying the condition of lymphadenoid goitre, first observed in human material by Williamson and Pearse (1925 ; 1929) and later produced in rats by McCarrison (1927–28, *a* ; 1927–28, *b* ; 1929–30, *a* ; 1930, *a*) by means of a vitamin-deficient diet. It will be shown that young rats fed on such a diet for some months developed simple goitre in spite of their receiving an adequacy of iodine, but in no case did there arise a lymphadenoid goitre such as these observers have described. McCarrison (1929–30, *a*) has drawn attention to the occurrence of cyst-like bodies in the thyroid and thymus glands of rats with lymphadenoid goitre ; these Williamson and Pearse (1930) interpret as being dilated lymph-channels, and cite them as evidence for the existence of a thyroid-thymic lymphatic connection. Similar bodies were obtained in the present series, but it will be shown that they are epithelial rather than endothelial in origin.

EXPERIMENTAL PROCEDURE.

The breed of rats used was the black and white pied variety of Norwegian rat, obtained from the Lister Institute through the kindness of Dr. Harriette Chick and Miss H. Smith. Four litters, 30 rats in all, were chosen ; the ages of those of two litters were 25 days and of the other two 26 days. Twenty-four of these, 9 males and 15 females, were taken for experimentation, and 6, 2 males and 4 females, for controls. Their weights on the day before the diet was begun ranged from 29 to 52 gm.

The rats were kept in a well-lit room, but never received the direct rays of the sun. They were housed in separate cages, which were scrubbed daily

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with an antiseptic lotion. A small amount of wood wool, changed daily, was used for bedding. The temperature of the room varied from 22·5° to 29·5° C.

Diet.

The diet adopted for the experimental rats was one by means of which McCarrison (1927–28, *a*) had produced lymphadenoid goitre in 33 per cent. of his animals. This consisted of white flour 82 per cent., olive oil 8 per cent., meat residue 5 per cent., salt mixture 5 per cent., and an unlimited supply of distilled water.

The meat residue was prepared as follows : Lean meat, from which all adherent fat was removed, was cut into thin strips and washed in many changes of water. It was then passed through a fine mincing machine and again washed in water, after which it was boiled for one hour in a 2 per cent. solution of sodium bicarbonate, drained, pressed, spread out on shallow trays and dried in a slow oven. It was then autoclaved at 130° C. for 1½ hours, and ground into a fine powder, when it was ready for use.

The salt mixture was made up as follows : Calcium lactate 39 gm., calcium phosphate 16·2 gm., potassium phosphate 28·62 gm., sodium phosphate 10·41 gm., sodium chloride 5·19 gm., magnesium sulphate 7·98 gm., iron citrate 3·54 gm., and potassium iodide 0·5 gm.

The principal fault of this diet lay in an insufficiency of vitamins A, B, C and D.

Controls.

The 6 control animals were also kept in separate cages, which were scrubbed with an antiseptic lotion twice weekly. They were bedded in wood wool, changed daily. Their diet consisted of dried winter milk, white bread, rice, oats, lentils, split-peas, fresh raw vegetables and tap-water. Marmite was mixed with food every day to give an abundance of vitamin B, and raw lean meat was given twice a week.

Histological Technique.

In order to avoid post-mortem autolysis as far as possible, immediately an animal was found dead or was killed, the larynx and trachea with the attached thyroid were removed (except in several animals to be considered forthwith), together with the pituitary, thymus, suprarenal glands and ovaries, and at once placed in fixative (Heidenhain's " Susa " solution). After fixation the thyroid was carefully dissected off the larynx and trachea. Serial sections were made of the various tissues, and were stained with Ehrlich's acid hæmatoxylin and azo-eosin.

Reference has already been made to the cyst-like structures observed in the thyroid and thymus of rats fed on a vitamin-deficient diet. If these structures represent varicose lymphatic channels between the thyroid and thymus, as Williamson and Pearse claim, one would expect to find similar cystic dilatations anywhere between the thyroid gland and thymic tissue in the neck or mediastinum. In 4 rats, therefore, the whole of the tissue enclosed between the pretracheal fascia anteriorly and the prevertebral fascia posteriorly, extending from the hyoid bone to the bifurcation of the trachea, was removed as a whole. This mass of tissue was embedded in paraffin, and transverse serial sections were made at various levels from the upper part of

the larynx to the bifurcation of the trachea. No greater distance than 100μ lay between each series of sections.

The thyroids of two experimental animals were fixed in 5 per cent. formol saline, but were not sectioned. In order to determine the conformation and connections of any cysts present, these glands were dissected under a dissecting microscope.

RESULTS.

General.

At the end of 2 months the experimental animals began to present the typical symptoms associated with vitamin deficiency. In a number ulceration around the mouth and anterior nares, a serous or sanguineous discharge from the nose, blepharitis and conjunctivitis appeared, and in several the whole eye became inflamed, leading to complete closure of the lids and blindness. Later, paresis of the hind limbs, believed to be a result of vitamin B deficiency, arose in two animals. A third exhibited the condition known as "twisting," characterized by alteration of equilibrium; the head was turned to the right, and when picked up by the tail the animal spun to the right. At autopsy the condition was found to be associated with a purulent infection of the right inner ear, and thus agrees with the observations of McCordock and Congdon (1924–25).

The rate of growth of the rats fed on the vitamin-deficient diet was considerably diminished. The greatest weights reached by the controls varied from 187 to 296 gm.; the greatest weights reached by the animals fed on the deficient diet for twelve months or more varied from 103 to 170 gm.

As one rat on the special diet died of pneumonia at the end of nine weeks and many of the others were in very poor condition, and as it was essential to keep them alive as long as possible, the vitamin-deficient diet was replaced by the ordinary standard diet for 3 days. Following this treatment the majority improved, but 3 died. If the rats died in this manner, the thyroid gland was useless for histology because of post-mortem autolysis. Thereafter, when asthenia ensued, the standard diet was given for 3 days or more, depending on the animal's condition. This happened in some cases once a month, in others once in about 2 months. If there was no improvement as a result of this treatment and death appeared imminent, the animal was killed by the inhalation of ether. Nevertheless, after being on the vitamin-poor diet for 6 to 11 months, 7 more rats were found dead. The remaining 13 were killed with ether at varying times between 3 and 13 months (2 after 3 months, 1 after 6 months, 1 after 11 months, 3 after 12 months and 6 after 13 months). Of the controls 1 died of pneumonia at the end of 10 months, and the remaining 5 were killed with ether at the end of 13 months.

Post-mortem Appearances.

General.

A variety of morbid states was encountered in the animals which were on the deficient diet. These consisted of the following in order of frequency: Purulent sinusitis and (or) suppurative otitis (14); inflammation of the eyes

(12); ballooning and atrophy of the intestines (10); enteritis (7); cystitis (3); vesicular calculi (3); pneumonia (2); broncho-pneumonia (2); peritonitis (2); pyelonephritis (1); dilatation of ureters (1); and suppurative meningitis (1). The frequent incidence of sinusitis and middle-ear infection was striking, and has been previously described in rats on a diet deficient in vitamin A by several observers (Daniels, Armstrong and Hutton, 1923; Sherman and Storms, 1925; Bradford, 1928).

The general post-mortem appearances of the control rats were normal, except in the one which died of pneumonia.

Thyroid Glands.

The thyroid glands of the 4 animals from which the bulk of the neck tissue was wholly removed could not be examined macroscopically, so that an examination was made in 20 of the 24 experimental animals. Goitre occurred in 15 of these, the remaining 5 thyroids being of normal size. The goitres were classified in the manner adopted by McCarrison (1930, *b*): (*a*) "Slight goitres," *i. e.* small enlargements amounting to less than twice the size of the normal gland, 8, of which 5 were but slightly enlarged and 3 definitely. (*b*) "Small goitres," *i. e.* moderate enlargements amounting to twice, but less than four times, the size of the normal gland, 5, of which 3 were twice as large and 2 three times. (*c*) "Large goitres," *i. e.* marked enlargements amounting to four or more times the size of the normal gland, 2, of which 1 was four times as large and 1 seven times. Usually the longer the animals were on the experimental diet, the more likely was a moderate or marked enlargement of the thyroid to appear. The 6 control rats exhibited no thyroid enlargement.

Thymus.

A feature in most animals fed on the vitamin-deficient diet was the great diminution of thymic tissue. This occurred in all animals except two, in which the thymus was much larger than normal. This degree of thymic atrophy was in agreement with the usual findings in animals fed on a diet deficient in vitamin A (Funk and Douglas, 1913–14; McCarrison, 1918–19 and 1919–20).

Histology.

Thyroid Glands.

Controls.—The glands of the control animals were normal. The vesicles on the whole were moderate in size, contained non-vacuolated well-staining colloid, and were lined with cubical epithelium. A few vesicles contained vacuolated, poorly staining colloid, and were lined with short columnar epithelium. Desquamated epithelial cells were occasionally seen.

Animals fed on the vitamin-deficient diet.—The outstanding feature of the thyroid glands of these rats was the occurrence of cyst-like structures in 23 of the 24. These will be described separately below. The gland which contained no cysts was normal in every respect. Of the 23 glands containing cysts, the general histological appearances were obscured in 5 by post-mortem autolysis, and were not studied in 2, in which a dissection of the cysts was

made. There were thus 16 abnormal glands for histological study. Two of these were normal in size, 4 were removed with the neck tissue, and the remaining 10 were goitrous. Their histological characters varied from colloid glands, in which evidence of activity was slight, to glands which were moderately hyperplastic. The animals had been on the experimental diet for periods varying from 3 to 13 months, and it appeared on the whole that the longer the animal had been on the diet, the greater was the tendency for its thyroid to be hyperplastic.

The colloid glands, of which there were 7, 3 enlarged and 4 removed with the neck tissue, differed very little from the normal. Most vesicles were fairly large, were filled with non-vacuolated, well-staining colloid, and were lined with low cubical or flattened epithelium. There were more desquamated epithelial cells than in the controls. Of the hyperplastic glands, 2 were normal in size and the remaining 7 were enlarged—3 slight, 2 small and 2 large goitres. In these glands there was a variable number of areas in which the epithelium was columnar ; in the more hyperplastic the lumen of groups of vesicles was obliterated on account of the tall columnar epithelium. There was also a greater number of vesicles containing desquamated epithelial cells, some being entirely filled with these cells and containing no trace of colloid. The interfollicular tissue was increased, but the lymphocytic aggregates described by McCarrison were lacking (Fig. 1). One gland which was moderately hyperplastic and between two and three times the normal size contained several extremely hyperplastic adenomata, in which the epithelium was tall columnar, and the lumen of the vesicles either completely obliterated, or reduced to a narrow irregular slit entirely devoid of colloid. Such adenomata are fairly common in goitrous rats (Wilms, 1910 ; Bircher, 1911).

Keratinization cysts.—These occurred in 23 of the thyroids, whether the gland was enlarged or not, and were identical with those produced by McCarrison (1929–30, *a*) in lymphadenoid and in simple goitre in rats. The fully developed cysts varied considerably in size, the largest being approximately 1·3 × 0·9 × 0·6 mm., and in shape, some being fairly round and regular, others irregular with numerous protuberances. The wall consisted of flattened cells resembling squamous epithelium and, externally, of a capsule of delicate fibrous tissue. Their contents consisted of whorls of a pale substance arranged in concentric laminæ, and staining either faintly basophilic, neutrophilic or eosinophilic (Fig. 2).

The various phases in the formation of these cysts could be traced from their inception to the final stage : (1) A number of sections contained an occasional vesicle in which all the colloid had disappeared and the lumen was filled with desquamated epithelial cells. The wall of such a vesicle consisted of either columnar or cubical epithelium. The appearance of epithelial cells within the lumen was not that occasionally simulated by the section shaving the wall of an irregular vesicle, for in all cases they filled the same vesicle throughout twelve or more sections of 7½μ each. Occasionally the colloid had not entirely disappeared, but was forced to one side by the mass of cells within the lumen. (2) In the next stage the vesicle was lined with low cubical epithelium, and contained a mass of epithelial cells degenerate in appearance. Within the centre of the mass was a faintly basophilic structureless material formed by

cell degeneration. In vesicles from which it had not wholly disappeared, the colloid assumed a whorled formation and mingled with the structureless material. (3) In a later stage the lining cells became more flattened and resembled squamous epithelium. Internal to them was occasionally a layer of scattered degenerate epithelial cells. In one gland the section shaved the wall of a cyst so that it was seen as a plaque ; it was composed of epithelial cells, not of pavement epithelium. The contents now consisted of a laminated, faintly basophilic or eosinophilic substance, with here and there a degenerate epithelial cell.

Large cysts were formed by the fusion of adjacent degenerate vesicles. One specimen showed a group of vesicles degenerating to form one large cyst : the colloid was disappearing, and what remained was beginning to assume a faintly whorled appearance. The epithelium of all the vesicles was desquamating into the lumen, and in some it was degenerating into a structureless substance, which mingled with the remnants of colloid. Some of the vesicles were already fusing. In some cases the cysts greatly contributed to the enlargement of the gland.

By studying the structures in series it was found that each cyst was complete in itself within the gland, and was not in communication with any lymphatic vessel.

Micro-dissection of cysts.—The four lobes of the two thyroid glands which were dissected contained cysts in each lobe. They were easily recognized by their white glistening appearance, and were readily dissected from the surrounding thyroid tissue. They were spherical or oval in shape, or, on the other hand, nodular and irregular. Their capsule was fairly tough and quite complete, there being no evidence that the cavity of the cysts was in communication with a lymphatic vessel.

Thymus.—The thymus glands of the majority of the animals which were on the special diet showed the marked lymphoid atrophy which is associated with vitamin-B deficiency. In 4 the thymus contained cystic spaces lined with ciliated columnar or cubical epithelium. Marine (1915) has drawn attention to these spaces, more especially in dogs, and has pointed out that they are probably embryological remains of the original thymic tubules (ducts of Remak). In 3 the thymus contained cyst-like structures similar in appearance to the final stage of those described in the thyroid, and varying from 0·2 to 0·5 mm. in diameter (Fig. 3). Situated in the medulla, they were lined in 2 cases with flattened cells with elongated nuclei, and contained a concentrically laminated eosinophilic hyaline material. In the third there was no definite cellular wall ; the structure consisted simply of the laminated substance surrounded by the lymphocytes of the medulla.

Trachea.

Of the 4 animals in which the whole of the neck tissue and superior mediastinum was examined in series, 3 showed metaplastic changes, consisting of keratinization of the epithelium lining the trachea and the laryngeal and tracheal mucous glands. This was associated with infiltration of mononuclear cells throughout the tracheal mucosa, so that it appeared thickened. The polypoid

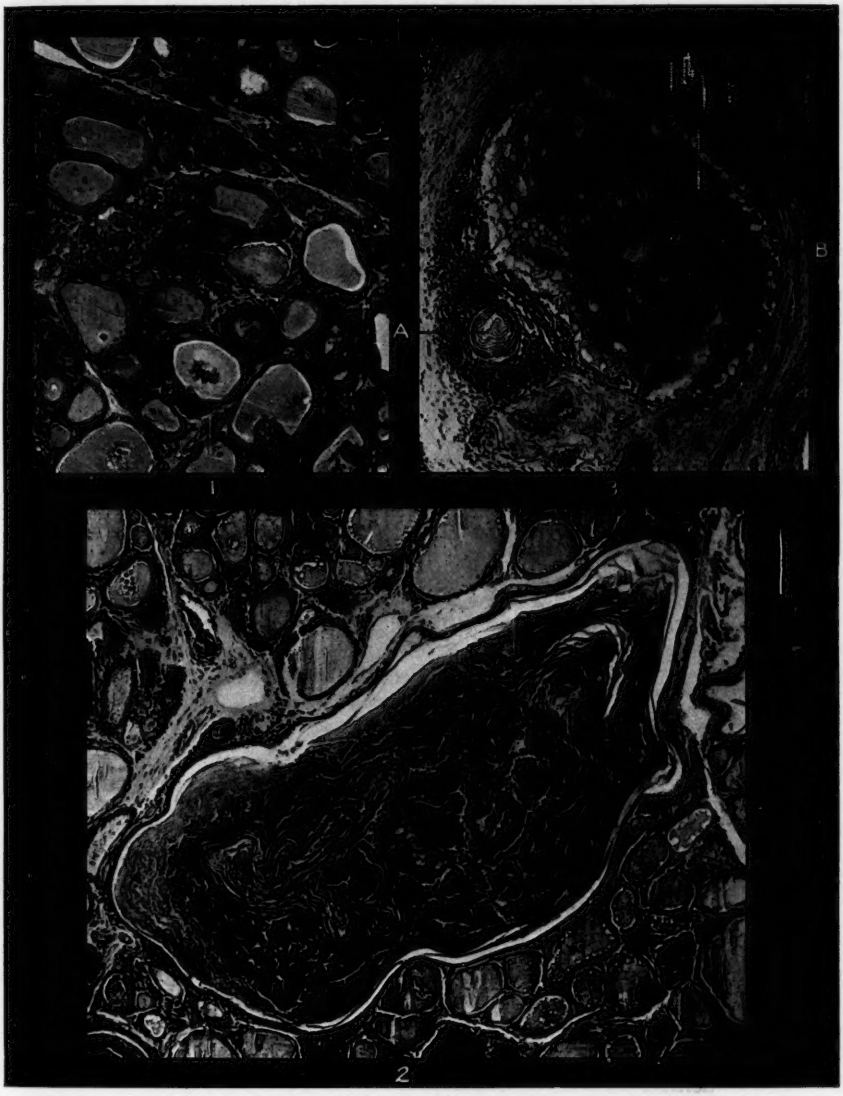

FIG. 1.—Simple goitre showing moderate hyperplasia produced in a rat by means of a vitamin-deficient diet. × 200.

FIG. 2.—A keratinization cyst of the thyroid gland (rat). × 200.

FIG. 3.—Thymus of a rat fed on a vitamin-deficient diet, showing atrophy, a large Hassall's corpuscle (A) and cystic remains (B). × 200.

projections of the tracheal mucosa described in this condition by McCarrison (1929–30, *b*) were not observed.

No keratinization cysts were discovered in the tissue between the thyroid and thymus.

Pituitary, Suprarenals and Ovaries.

These were examined in 7 rats which had been on the diet for periods varying from 2 to 6 months. Stained with Ehrlich's acid-hæmatoxylin and azo-eosin the organs appeared normal.

DISCUSSION.

The significance of the thyroid changes.—Fifteen of the 20 glands which were examined macroscopically were enlarged, and these showed various phases of activity from the resting colloid stage up to moderate hyperplasia. In spite of adopting an identical technique, the lymphadenoid type of goitre produced by McCarrison was not obtained. To account for the association of goitre with vitamin deficiency and an adequacy of iodine, McCarrison (1929–30, *a*) has suggested that such a diet may cause thyroid disorder in one or all of the following ways : " (1) By inducing a state of imperfect nutrition of the thyroid gland with consequent lowering of its physiological efficiency ; (2) by disturbing the normal processes of metabolism with the control of which the thyroid gland is so closely concerned ; (3) by rendering the organ liable to attack by bacterial agents or their products derived from such situations as the upper alimentary tract." It has been seen that many of the animals in the experiments described presented evidence of some degree of chronic sepsis. The number of animals with infection of the middle ear and communicating sinuses or with intestinal atrophy and enteritis is too large to be overlooked. Such a stimulus is recognized as an indirect cause of goitre. Vitamin deficiency itself may give rise to thyroid enlargement, for it occurred in a few rats in which there was no evidence of sepsis. When, however, infection is superimposed on such a profound dietetic insufficiency, the liability to the occurrence of thyroid hyperplasia is increased.

Keratinization.—An outstanding feature was the extremely frequent incidence of keratinization cysts in the thyroid gland of the rats on the vitamin-deficient diet, and the occurrence in several cases examined of keratinization of the epithelium lining the trachea and laryngeal and tracheal mucous glands. Wolbach and Howe (1925 and 1928) have shown that keratinization of epithelial tissues is a specific effect of vitamin-A deficiency. They described it in the conjunctiva, the cornea, the respiratory and genito-urinary tracts (including the ovary and uterus), the pancreas, the thymus and the para-ocular, submaxillary and parotid glands. Seifried (1930) produced it in the respiratory tract of chickens by means of a vitamin-A deficient diet, and noted the secondary infections that were often found in conjunction with it. McCarrison was the first to demonstrate keratinization in the thyroid.

That the cysts in question may be cystic rests corresponding to the ultimobranchial body or that they may be thyroglossal cysts has not been overlooked. However, by a study of what are considered to be the various stages in their formation, it has been shown above that they undoubtedly arise from the thyroid vesicle. It is concluded that the factor determining their formation

is exhaustion of the vesicular epithelium, already in a physiologically subnormal state as a result of deprivation of vitamins, and consequently failing to cope with the demands made upon it—demands which lead to hyperplasia of the thyroid, and now later to exhaustion of its morphological elements. Williamson (personal communication), in an examination of about 7000 human thyroids, has never seen such structures in man.

Cysts of a similar appearance occurred in the thymus of three animals on the special diet. Williamson and Pearse (1930) correlate the presence of these bodies in the thymus with the cysts in the thyroid, and suggest that both represent a varicose condition of the thyroid-thymic lymphatic connections, the result of increased functional activity due to increased lymphogenesis of the endothelial elements of the thyroid-thymic lymphatic system. In the present experiments there was no evidence of increased lymphogenesis in the thyroid, and yet cysts were observed in 23 of the 24 glands. If these structures were lymphatic channels, one would expect their formation to run parallel with the production of lymphocytes. Moreover, one would also expect to find evidence of similar structures lying in the tissue intervening between the thyroid and thymus, and their occurrence in a larger percentage of thymus glands in this condition. Such was not the case in the present series. I consider that the presence of these bodies in the thymus is not an effect of vitamin deficiency, but that they are merely large Hassall's corpuscles. That Hassall's corpuscles may be cyst-like in appearance and may have a diameter as great as 1 mm. is well known (Marine, 1915).

SUMMARY.

1. An account is given of an attempt to produce lymphadenoid goitre in a batch of 24 young rats by means of a diet consisting of white flour 82 per cent., olive oil 8 per cent., meat residue 5 per cent., salt mixture (including an adequacy of potassium iodide) 5 per cent., and an unlimited supply of distilled water. The animals were on the diet for periods varying from 9 weeks to 13 months.

2. Simple goitre was produced in 15 out of 20 rats examined, of which 8 had slight goitres, 5 small goitres, and 2 large goitres. Lymphadenoid goitre did not arise in any rat.

3. The production of simple goitre was independent of an absolute iodine deficiency. The factors concerned in its production were twofold: dietetic insufficiency and infection.

4. An outstanding feature was the occurrence of keratinization cysts in the thyroid in 23 of the 24 animals. It is shown that these cysts arose from the vesicles, probably as a result of exhaustion.

5. Similar cysts were found in the thymus of 3 animals on the diet, and are considered to be large Hassall's corpuscles, and not an effect of vitamin deficiency.

6. No evidence was found in support of the hypothesis that the cysts in the thyroid and thymus represent distensions of the thyroid-thymic lymphatics.

To Prof. H. Hartridge I owe my thanks for his critical interest, for permitting me to carry out this work in the laboratories under his care, and for

a grant towards the expenses of the research. Further, I wish to record my indebtedness to Prof. F. R. Fraser, Prof. H. Woollard, Dr. G. Scott Williamson, Dr. Innes H. Pearse and Mr. F. Haynes for their advice, and for the interest they have taken in this research, and also my appreciation of the services of Mr. W. Barrett, who was responsible for the feeding and care of the animals. I wish to thank Dr. E. A. Carmichael for his help with the micro-photographs. The work was conducted under the tenure of the Lawrence Research Scholarship, and with the aid of personal and expenses grants from the Medical Research Council.

REFERENCES.

Bircher, E.—(1911) *Deuts. Z. Chir.*, **112**, 368.
Bradford, W. L.—(1928) *J. Infect. Dis.*, **43**, 407.
Daniels, A. L., Armstrong, M. E., and Hutton, M. K.—(1923) *J. Amer. Med. Ass.*, **81**, 828.
Funk, C., and Douglas, H.—(1913–14) *J. Physiol.*, **47**, 475.
McCarrison, R.—(1918–19) *Ind. J. Med. Res.*, **6**, 284, 550.—(1919–20) *Ibid.*, **7**, 269, 308.—(1927–28, *a*) *Ibid.*, **15**, 247.—(1927–28, *b*) *Ibid.*, **15**, 909.—(1929–30, *a*) *Ibid.*, **17**, 442.—(1929–30, *b*) *Ibid.*, **17**, 693.—(1930, *a*) *Ibid.*, **18**, 577.—(1930, *b*) *Brit. Med. J.*, **1**, 989.
McCordock, H. A., and Congdon, C. C.—(1924–25) *Proc. Soc. Exp. Biol. Med.*, **22**, 150.
Marine, D.—(1915) *Cleveland Med. J.*, **14**, 186.
Seifried, O.—(1930) *J. Exp. Med.*, **52**, 519.
Sherman, H. C., and Storms, L. B.—(1925) *J. Amer. Chem. Soc.*, **47**, 1653.
Williamson, G. S., and Pearse, I. H.—(1925) *J. Path. Bact.*, **28**, 361.—(1929) *Brit. Med. J.*, **1**, 4.—(1930) *Brit. J. Surg.*, **17**, 529.
Wilms, M.—(1910) *Deuts. med. Wschr.*, **36**, 604.
Wolbach, S. B., and Howe, P. R.—(1925) *J. Exp. Med.*, **42**, 753.—(1928) *Arch. Path.*, **5**, 239.

THE DEMONSTRATION OF HERPETIC ANTIBODY IN HUMAN SERA BY COMPLEMENT-FIXATION, AND THE CORRELATION BETWEEN ITS PRESENCE AND INFECTION WITH HERPES VIRUS.

R. T. BRAIN.

From the Freedom Research Laboratory, London Hospital.

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It is known that a considerable percentage of human sera possess the power of neutralizing herpes virus. Both Zinsser and Tang (1929) and Andrewes and Carmichael (1930), who have made observations on this point, were concerned principally with finding out whether the incidence of this specific neutralizing power was greater in patients convalescent from encephalitis

lethargica than in normal people. And although their findings revealed no significant difference between the two groups, they show that a high percentage of human sera are capable of neutralizing herpes virus. Their findings can be summarized as follows :

Author.	Source of serum.	Number.	Percentage giving neutralization.
Zinsser and Tang	Encephalitis lethargica	47	87
	Normal adults	17	58
	,, children	23	43
Andrewes and Carmichael	Encephalitis lethargica	25	72
	Normal adults	28	75

Apparently no attempt was made to correlate these findings with evidence of herpetic infection as revealed by the presence, or history, of the characteristic vesicular eruption caused by this virus. This is an important point, because without this information it is impossible to estimate the significance of the finding of herpetic antibody in human sera. Should the presence of this antibody occur only in individuals who were subject to herpetic eruptions, then its demonstration would be of use not only in helping occasionally to arrive at a clinical diagnosis, but also in throwing some light on the carrier rate of herpes virus in man, and providing additional evidence of the extrinsic nature of herpes virus. This question has therefore been investigated, and the findings form the subject of this paper.

EXPERIMENTAL DATA.

Choice of Method.

It has been shown by Bedson and Bland (1929) that with suitable technique, specific complement-fixation can be obtained with herpes virus in the presence of an antiherpetic serum. They worked with a saline suspension of herpetic guinea-pig "pads" as antigen, and hyper-immune sera made in that animal, and since some preliminary observations made with unselected human sera showed that a considerable number gave specific fixation in the presence of a guinea-pig herpes antigen, it was decided to use this reaction as the test for presence of herpetic antibody. It is true that in the case of antiherpes sera prepared in the guinea-pig the neutralizing titre is greater than the complement-fixing titre. Bedson and Bland (1930) observed this, and a considerable number of parallel titrations made with hyperimmune guinea-pig serum during the course of this work have borne this out. The fact, too, that of an unselected series of 51 human sera examined for herpes antibody by complement-fixation, only 23, or 45 per cent., gave a positive result—a lower figure than either Zinsser and Tang (1929) or Andrewes and Carmichael (1930) obtained using the neutralization test—suggests that with human sera also the complement-fixation reaction is the less sensitive. However, this reaction is easy to carry out, and requires neither the time nor the expenditure in animals which the neutralization experiment entails, and though admittedly a slightly less delicate indicator of herpes antibody, it is a reliable one, provided that a suitable technique is employed.

Technique of the Test.

The test employed has been essentially that described by Bedson and Bland (1929).

Antigens.—The antigen used has been a suspension of herpetic guinea-pig pads in M/50 phosphate buffer pH 7·6, and the same strain of herpes has been used throughout. This strain was isolated from typical labial herpes on October 29th, 1930, and has been maintained in guinea-pigs since that date, having undergone 75 passages. Attempts were made to improve on this antigen. The cloudiness which remains in phosphate pad suspensions after centrifugation to throw down coarse tissue particles can be removed by ether extraction, but the resulting antigen is not as satisfactory as the original suspension. A dry stable antigen would be a distinct advantage, but when herpetic pads were dried *in vacuo* over $CaCl_2$ and the dried powder suspended in phosphate some two months later, the suspension was found to be of no value as an antigen. In all tests a control antigen was used. This was a phosphate suspension of normal guinea-pig pads prepared in exactly the same way as the herpetic one. It was noticed quite early on that a freshly-prepared herpes antigen did not give such good fixation as one which had been kept for a few days in the refrigerator, and although this observation has been acted on, the underlying cause for the phenomenon remains obscure.

Before use the antigens have been tested invariably for any anti-complementary power by titration in the presence of 1 M.H.D. of complement. Any antigen which inhibited in any degree the action of 1 M.H.D. of complement when used in double the quantity to be used in the test was discarded. This titration of anti-complementary power was determined on the day of the test.

Sera.—The human sera to be examined were used undiluted, and heated for 20 minutes at 56° C. In addition to the unknown sera, each test included the guinea-pig antiherpes serum of known fixing potency and a known negative human serum.

Controls.—In addition to the controls already mentioned, each test carried the usual control tubes containing (1) antigen and complement (2), unknown serum and complement, (3) complement alone.

Evidence of Herpetic Infection.

Positive evidence in many instances depended on personal observation of the herpetic eruption; in some cases, however, history of a herpetic eruption has been accepted, but only where it was known to be entirely reliable. A negative history of herpetic infection is a much more difficult matter to be certain about. In the case of the ordinary hospital patient it is quite impossible to rely on the history, and here use has been made of the observation that protein shock therapy, in the form of T.A.B. vaccine intravenously, is accompanied by an outbreak of labial herpes in those subject to this condition. Patients, therefore, who gave a negative herpetic history and who developed no herpes following protein shock therapy have been accepted as negative. The majority in the negative group, however, were colleagues whose history in all probability could be relied on.

Results.

A possible fallacy in this test might conceivably be that sera giving a positive Wassermann reaction might fix complement in the presence of the herpetic antigen, which, of course, is a suspension of tissue. This point was examined, but the following figures show that herpetic fixation has nothing to do with the Wassermann reaction of the serum:

Wassermann reaction.		Fixation with herpes antigen.	
+	−	+	−
13	..	5	8
..	38	18	20

The results of a typical test are given in Table I.

TABLE I.—*Complement 2 M.H.D. Fixation for 2¾ Hours at Room Temperature.*

Sera.	Antigens. Herpes-pad suspension.	Normal pad suspension.	Saline.
Guinea-pig. Antiherpes B. 371. 1 in 20	++++	−	−
	+++	−	−
Guinea-pig. Antiherpes S. 1. 1 in 10	++++	−	−
	++++	−	−
Human. Recurrent herpes	++++	+	−
	+++	−	−
Human. No herpes	−	−	−
	−	−	−
Saline	−	−	−
	−	−	−

In this and subsequent tables the degree of fixation is indicated by ++++ +++ . . . . . ± −. In each pair of readings the upper was made after 30 minutes at 37° C., the lower after standing overnight.

When human sera are somewhat anti-complementary in spite of heating, fixation with 2 M.H.D. of complement may occur with the control normal pad emulsion, but the non-specific character of such fixation can usually be shown by repeating the test with 3 or 4 M.H.D. of complement. Table II illustrates this well.

Out of 95 human sera examined, only 48 were from individuals in whom reliable evidence as to herpetic infection was obtained. Thirty-two of these were individuals with either herpetic lesions or reliable histories of infection with *herpes febrilis*; the remaining 16 had no history of herpes. These 16 include 7 normal individuals and patients with the following diseases: Psoriasis, dermatitis herpetiformis, weather dermatitis (2), sycosis barbæ, urticaria,

TABLE II.—*Fixation for 2½ Hours at Room Temperature.*

Sera.	Antigens. Herpes pad suspension. 2 M.H.D. complement.	Herpes pad suspension. 3 M.H.D. complement.	Normal pad suspension. 2 M.H.D. complement.	Normal pad suspension. 3 M.H.D. complement.	Saline. 2 M.H.D. complement.
Guinea-pig. Antiherpes S. 1. 1 in 10	++++	..	−	..	−
	++++	..	−	..	−
Human No. 71. No herpes	++++	±	++++	−	−
	++	−	+	−	−
Human No. 72. History of herpes	++++	++++	−	−	−
	+	+	−	−	−
Human No. 73. History of herpes	++++	++++	++++	−	−
	+++	+	+	−	−
Human C.P. 2. No herpes	++++	−	++++	−	−
	−	−	−	−	−
Saline	−	..	−	..	−
	−	..	−	..	−

toxic erythema, renal calculus and chronic arthritis. The results of complement-fixation tests with these 48 sera in the presence of a herpetic antigen are as follows :

Source of serum.	Number.	Fixation with herpes antigen. +	Fixation with herpes antigen. −
Individuals with herpetic lesions or a reliable history of herpetic infection	32	30	2
Individuals with a negative history of herpes	16	0	16

The two individuals with a positive history who furnished negative sera were both cases of recurrent herpes, but in which no eruption had occurred for three months. Against the view that this interval was responsible for the absence of fixation with a herpes antigen, may be placed the observation that positive fixation occurred with the sera of two individuals in whom there was an interval of 6 months and 1 year respectively between the collection of serum and the last outbreak of herpes. So that apart from the suggestion that these two individuals were poor antibody formers, no explanation can be offered for the two negative results. Two sera from cases of recurrent herpes were tested repeatedly, one 13 and the other 6 times, and on every occasion positive fixation was obtained. One known negative serum gave 13 successive negative fixations. Reference has been made to the fact that in certain cases it had been possible to check the herpetic history by the response to protein shock. This was done in 10 cases, and the findings in them are as follows :

Herpetic history.	Number.	Herpes following protein shock.		Fixation with herpes antigen.	
		−	+	−	+
Negative	6	6	0	6	0
Positive	4	1	3	0	4

Apparently protein shock cannot be relied on to light up herpes in the case of every individual subject to herpes. Although the clinical diagnosis of herpes febrilis is rarely difficult, it would appear that the complement-fixation test may be of service occasionally in supplying reliable confirmation. It proved of value, for instance, when a surgical colleague sought an opinion on vesicular eruptions, which resembled zoster in that they appeared on skin areas corresponding to cranial nerves which had been subjected to surgical intervention. Four cases were investigated, and all gave fixation with a herpes, but not with a zoster antigen. The question arises as to the bearing of the incidence of herpes antibody in human sera on the carrier rate of herpes in man. It has been shown (Levaditi, Harvier and Nicolau, 1922; Busacca, 1924–5) that herpes carriers exist, and clinical experience certainly supports this view. It seems difficult to believe that a trivial and transient lesion such as herpes, even when repeated at intervals as in recurrent herpes, could give rise to the persistent presence of herpes antibody in the blood. But the phenomenon finds a ready explanation if we assume that herpes sufferers are also carriers of the virus, for then there would be a constant stimulus to antibody production. If this be accepted, then the incidence of herpes antibody in man is an index of the carrier rate of herpes virus.

CONCLUSIONS.

1. A considerable number of human sera give specific complement-fixation in the presence of a herpes antigen.
2. Out of 52 unselected sera, 23, or 45 per cent., gave fixation.
3. This fixation bore no relation to the Wassermann reaction.
4. Examination of 48 selected sera from individuals in which the herpetic history was known shows that positive fixation is definitely related to herpetic infection.

Grateful anknowledgment is made to Dr. S. P. Bedson for much guidance and help in this work, which was assisted by a grant from the London Hospital Research Funds.

REFERENCES.

ANDREWES, C. H., AND CARMICHAEL, E. A.—(1930) *Lancet*, **1**, 857.
BEDSON, S. P., AND BLAND, J. O. W.—(1929) *Brit. J. Exp. Path.*, **10**, 393.
BUSACCA, A.—(1924–5) *Arch. Augenheilk.*, **95**, 253.
LEVADITI, C., HARVIER, P., AND NICOLAU, S.—(1922) *Ann. Inst. Past.*, **36**, 63, 105.
ZINSSER, H., AND FEI-FANG TANG (1929) *J. Immunol.*, **17**, 343.

SPONTANEOUS FLUCTUATIONS IN THE RETICULOCYTE COUNT IN PIGEON'S BLOOD.

LUCY WILLS.

From the Pathological Department, Royal Free Hospital, and Physiology Department, London School of Medicine for Women.

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VAUGHAN, in an interesting paper (1930), has described the use of pigeons for testing the hæmopoietic potency of various liver fractions, using the rise in the percentage of reticulocyted red cells as an index of activity. As a necessary preliminary, she showed that on a diet of mixed grains and beans certain pigeons maintained steady weights and reticulocyte counts not above 12 per cent. Birds that remained steady for a short control period after feeding for some weeks on the standard diet were considered suitable for test purposes.

With a view to determining what fraction of marmite is the active factor in the treatment of tropical macrocytic anæmia (Wills, 1931), an attempt was made to repeat Vaughan's work. This was not successful, possibly owing to the limited number of birds used, but the work is reported, as it demonstrates a few of the difficulties that may arise.

EXPERIMENTAL.

Vaughan's methods were used throughout. The birds were kept as far as possible under standard conditions of heat, water supply, etc., and were fed on mixed grains as described. Chart 1 may be taken as representative of a group of ten carrier pigeons that were originally tested. At A is seen the initial high percentage of reticulocytes reported by Vaughan, as usual in birds when first received from the market. From A to A^1 the conditions remained the same, but at A^1, though the reticulocyte count and the weight curve had remained steady for 20 days, the reticulocyte count rose suddenly to 19 per cent., a rise of the order taken as indicating a response to an active liver fraction, but occurring here in an untreated bird. At the same time three other pigeons whose reticulocyte counts had remained steadily below 10 per cent. for a fortnight were treated, one with liver extract, two with marmite. The one on liver extract, and one of the two on marmite, showed a rise in the reticulocyte count to 30 per cent., the remaining one on marmite to 18 per cent. only. Another control bird also showed a sudden and unaccountable reticulocyte rise to 25 per cent. As the rises in treated and untreated birds were of the same order, it was obvious that no conclusion would be drawn from such results. At this stage certain of the birds were fed on polished rice for 4 days ; the period B–B^1 shows the immediate rise of the reticulocyte count to 40 per cent. in the particular bird charted. Pigeon 5, which had previously received liver extract, had a reticulocyte count of 60 per cent. on the third day of the rice diet.

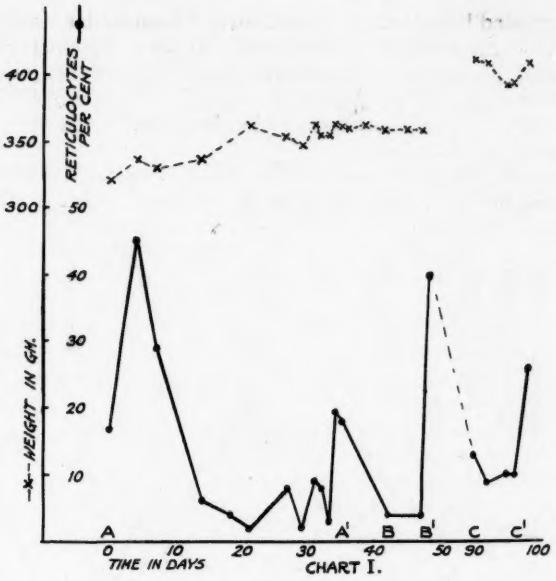

CHART I.

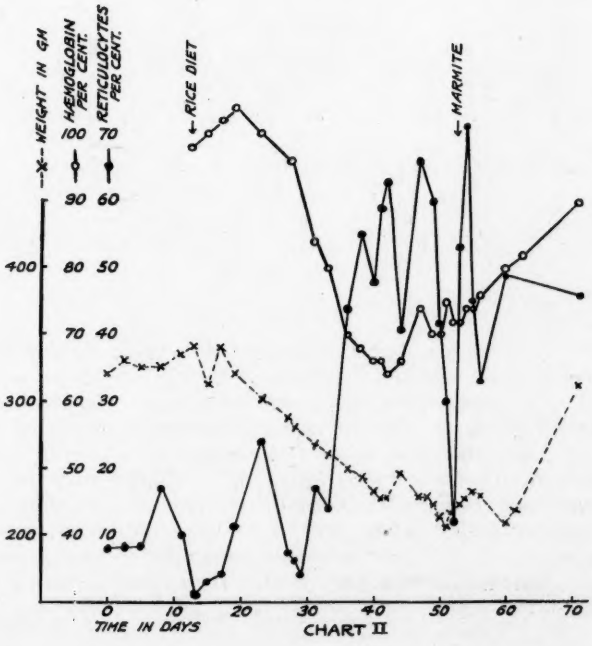

CHART II

It was suggested here that the birds had not been under standard conditions long enough to become stable, so they were left for a further period of 42 days. The period C–C^1 shows the reticulocyte range during the following 8 days. It is obvious that the birds were still unstable as regards their reticulocyte counts. As the birds had been under standard conditions for over 3 months, the experiment was abandoned.

The matter was next approached from another angle. Barlow and Biskind (1928, 1930) have shown that pigeons on a diet of polished rice, or of water

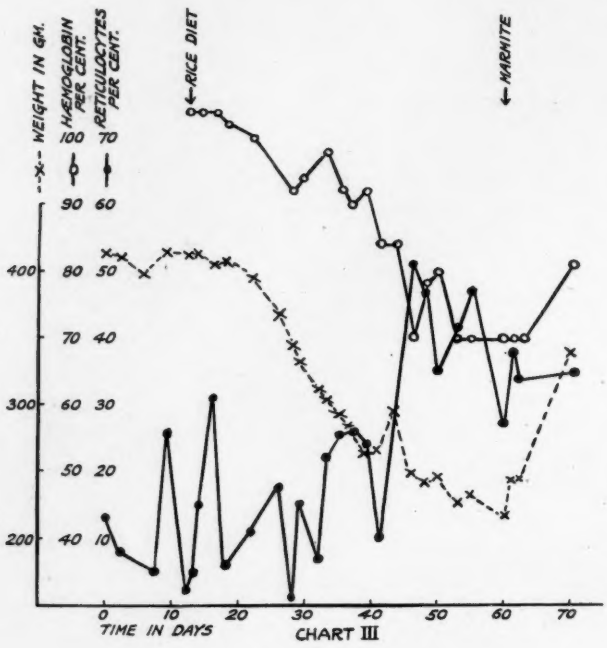

CHART III

only, not only lose weight, but at the same time show a progressive anæmia which can be cured by yeast or yeast extracts. Further, Doan and co-workers (1925) have shown that the bone-marrow of starving pigeons becomes hypoplastic. It was therefore thought possible that such rice-fed pigeons might, after the first initial rise, have steady, low reticulocyte counts, and that if so, they would be suitable for test purposes. Charts 2 and 3 are representative of a group of seven birds on a polished rice diet. The birds were kept on the diet till they developed severe polyneuritis, when they were given marmite to save them. The charts show very clearly that such rice-fed pigeons are even more unstable as regards their reticulocyte counts than birds fed on the mixed grain diet.

CONCLUSIONS.

These two sets of observations illustrate the difficulties in the way of using pigeons for experimental work, when the activity of a food or a drug for blood formation is to be judged by the reticulocyte response to the same. In a personal communication, Vaughan stated that only a limited number of her birds were found suitable, and that the experimental conditions, such as temperature and water supply, had to be kept within very narrow limits. The fact that in the experiments reported above the reticulocyte counts in certain birds remained under 10 per cent., and the weights steady for periods up to 3 weeks—her standard conditions—and then fluctuated for no discoverable reason, indicates the difficulties and the need for very large numbers of controls. A further difficulty is the fact that in the present series any change in the diet, either advantageous or the reverse, produced a rise in the percentage of reticulocytes, and that with a steadily increasing anæmia there was a marked increase in the percentage of reticulocyted reds, so that it was impossible to judge the response to any drug by the number of such present. Obviously the variations in the percentage of reticulocytes in pigeons depend on other factors than those operating in man, and the greatest caution is therefore necessary in the interpretation of results.

In conclusion I wish to thank Mr. J. Whitaker for his skilful help with the birds.

REFERENCES.

BARLOW, O. W., AND BISKIND, M. S.—(1928) *Amer. J. Physiol.*, **86**, 594.—(1930) *Ibid.*, **93**, 161.
DOAN, C. A., CUNNINGHAM, R. S., AND SABIN, F. R.—(1925) *Contributions to Embryology*, **16**, 83, 165.
VAUGHAN, J. M., MULLER, G. L., AND ZETZEL, L.—(1930) *Brit. J. Exp. Path.*, **11**, 456.
WILLS, L.—(1931) *Brit. Med. J.*, **1**, 1059.

ON THE TECHNIQUE OF EXPOSING THE CHORIO-ALLANTOIC MEMBRANE OF THE CHICK EMBRYO FOR EXPERIMENTAL PURPOSES.

DAPHNE GOULSTON, B.Sc., AND J. C. MOTTRAM, M.B.

From the Pathological Research Laboratory, Mount Vernon Hospital, Northwood, Middlesex, and the Radium Institute, London.

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THE allantoic membrane lining the shell membrane of the chick embryo has been largely used for research, for instance, for the study of grafts of cancer

and of other tissues implanted into it, and for investigations upon the biological action of X-rays and radium. We have in mind especially the important works of Moppett (1931), who finds that X radiations of different wave-lengths produce widely different biological reactions. Having decided to use the allantoic membrane for radiological investigation, we commenced by seeing whether the technique of exposing it by removing a portion of the egg-shell gives rise to any changes in it.

TECHNIQUE EMPLOYED.

In order to expose the chorio-allantoic membrane, a portion of the egg-shell was removed by making three saw-cuts, 0·5 cm. apart, then three similar cuts at right angles to these, so that four pieces of shell were isolated each $\frac{1}{4}$ sq. cm. in area; these four pieces were then gently eased up with a scalpel, and picked off with forceps, leaving a square hole in the egg-shell of 1 sq. cm.; thus the shell membrane was exposed, beneath which lies the allantoic membrane.

In order to reproduce the conditions under which exposures to radiation would be made, these windows were left exposed to the air in the incubator for 2 hours, then covered with a sterile piece of egg-shell and sealed down with paraffin wax.

GENERAL DESCRIPTION OF FINDINGS.

Eggs treated in the way described, that is, as if they were being exposed to radiation, but without any radiation, often showed changes in the allantoic membrane where the window had been cut. To the naked eye the membrane, if abnormal, appeared sometimes thickened and sometimes thinned. When it was thinned, the membrane was more transparent than normal, and the blood-vessels were absent. When it was thickened the membrane was more opaque than normal. Small petechial hæmorrhages sometimes occupied the window, but they have also been seen in other parts of the membrane. Histologically these were recent hæmorrhages, and probably occurred when the embryo was being removed from the egg on the 13th day.

In order to study the histological changes, the membrane was fixed in either Bouin's solution or Bles's solution, and stained in various ways, of which iron-hæmatoxylin gave the best results.

The normal allantoic membrane is shown in Fig. 1. The width of the mesoderm varies widely, otherwise the membrane does not show variation except that the blood capillary close to the shell membrane may lie either within or without the ectodermal layer; when it lies without, its endothelial cells (D) are seen, as shown in the figure, to be directly in contact with the shell membrane.

When the membrane at the window presents the transparent bloodless condition previously described, sections show that it is dead and disorganized, so that none of its normal structure is recognizable, and only a thin layer of necrotic cells remains to be seen (see Fig. 5, A). When the membrane at the window is thickened, microscopical examination reveals hypertrophy of the

different layers. There may be thickening of the ectoderm, as shown in Fig. 2. In this case we have noticed that the capillaries immediately underlying the shell membrane have suffered injury, being no longer recognizable, and only represented by degenerated and shrivelled red cells, as shown in Fig. 2.

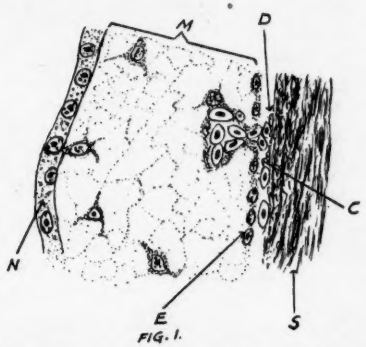

FIG. 1.

Fig. 1.—Normal allantoic membrane on 13th day. N = Endoderm; E = ectoderm; C = capillary; S = shell membrane; M = mesoderm; D = endothelial cell.

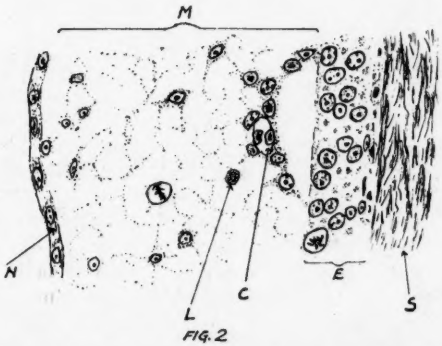

FIG. 2

Fig. 2.—Abnormal allantoic membrane on 13th day, showing ectodermal hypertrophy. N = Endoderm; C = capillary; L = lymphocytes; E = hypertrophied ectoderm; S = shell membrane; M = mesoderm. Note absence of egg-shell capillary, now represented by a few degenerated red cells and cell-*débris*.

In other cases the mesoderm is also hypertrophied, as shown in Fig. 3; here not only is the thickness of the mesoderm increased, but it is also more cellular, being invaded by cells of various kinds, fibroblasts, macrophages, lymphocytes, rarely polymorphs and hæmatopoietic cells. Blood-vessels and capillaries are also more numerous than normally. When the mesoderm

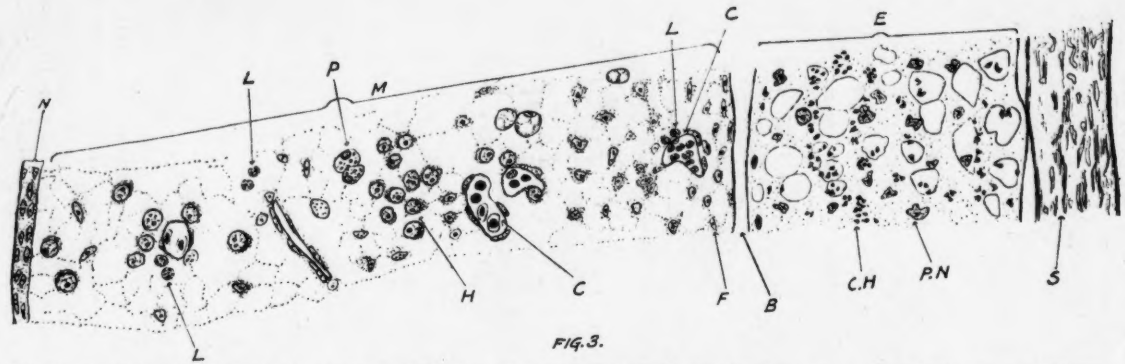

FIG. 3.

FIG. 3.—A thickened allantoic membrane on 13th day, showing mesodermal hypertrophy. N = Endoderm; E = ectoderm showing hypertrophy and necrosis; C = capillaries—note absence of capillary next to shell membrane; S = shell membrane; M = mesoderm, greatly thickened; L = lymphocytes; H = hæmatopoietic cells; P = macrophages; F = fibroblasts; CH = chromatin granules; PN = pyknosed nuclei; B = break between dead ectoderm and living mesoderm.

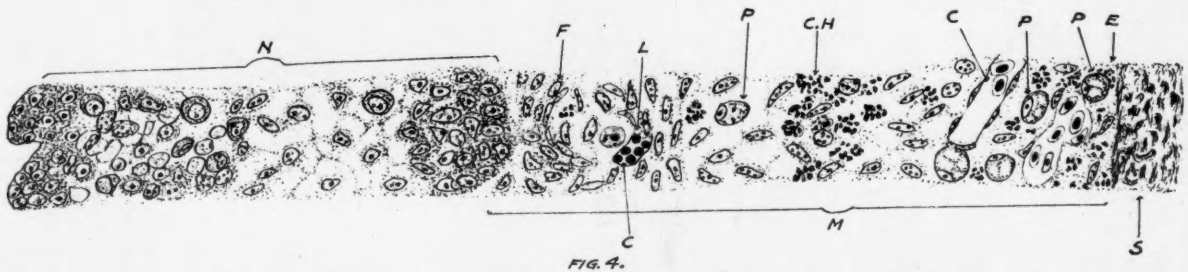

FIG. 4.

FIG. 4.—A thickened allantoic membrane on the 13th day, showing endodermal hypertrophy. N = Endoderm, here greatly thickened; E = the position of the ectoderm, it and the egg-shell capillary are no longer to be recognized; C = capillaries; S = shell membrane; M = mesoderm, greatly thickened and showing necrosis in its outer half; L = lymphocytes; P = macrophages, in many cases containing cell-*débris*; F = fibroblasts, chiefly close to the endoderm; CH = chromatin granules.

presents this hypertrophy the ectoderm is generally necrotic, though evidence of previous hypertrophy is usually to be seen (this is also shown in Fig. 3).

Lastly hypertrophy of the endoderm occurs. This hypertrophy varies from a layer of a few cells thick, to an increase of a bulky solid outgrowth into the allantoic cavity, or a solid downgrowth of cells into the mesoderm, or both. A typical hypertrophy is shown in Fig. 4.

As with the hypertrophy of the mesoderm, the presence of necrosis of the ectoderm, and often, in this case, of the mesoderm as well, has been observed, as shown in Fig. 4. In cases where necrosis of the mesoderm is not seen, the

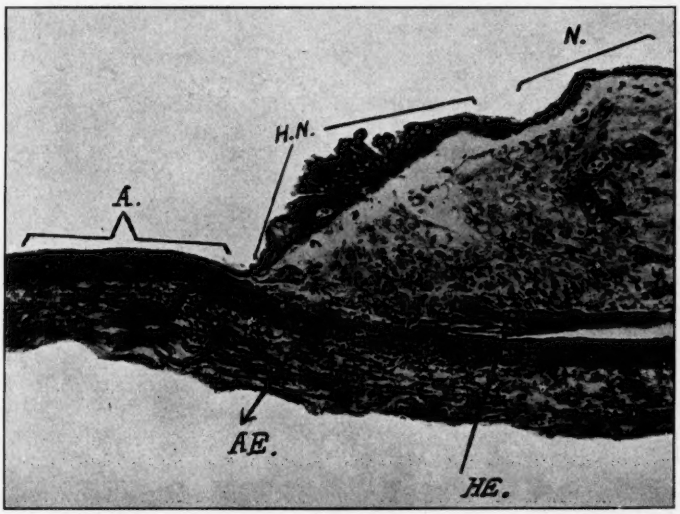

FIG. 5.—Photograph (× 250) of the junction of atrophic and hypertrophic parts of an abnormal allantoic membrane. A = Atrophic portion of membrane; AE = necrosed or atrophic ectoderm; HE = hypertrophied ectoderm; HN = hypertrophied endoderm; N = normal endoderm.

blood-vessels of the mesoderm show evidence of injury, being thrombosed, and containing altered and degenerated red cells.

It sometimes happens that one portion of the allantoic membrane shows at the window hypertrophy, and another portion the thinned and completely dead condition; where these two different conditions meet, a peculiar reaction of the membrane is found, which is of common occurrence. This is shown in Fig. 5, upon which we put the following interpretation:

On the left is seen the completely dead membrane (A). As we pass towards the living membrane on the right, there is seen hypertrophy of the endoderm (HN) for a short distance, and this then becomes normal (N). The mesoderm shows a gradually increasing hypertrophy (as shown by an increased cellularity), which, like the endoderm, fades into a normal mesoderm, not seen in

the figure. The ectoderm is at first necrotic and absent (AE); then a section of hypertrophy (HE) is seen, and further still to the right it becomes (not in the figure), like the other layers, normal.

Summarizing these histological findings, we conclude that the changes represent reactions to injury. When the injury is so slight as only to involve the capillaries lying immediately beneath the shell membrane, the ectoderm hypertrophies; when the ectoderm is also involved, the mesoderm hypertrophies; and when the mesoderm also suffers injury, then the endoderm hypertrophies. When all these layers are injured, then no hypertrophy occurs, the membrane is disorganized and has the appearance of being atrophic.

We therefore conclude that all these changes are reactions to injury, varying in intensity.

EXPERIMENTAL DETAILS.

Three sets of experiments have been carried out in order to discover how to avoid these injurious effects. In one set the window was made on the 10th day of incubation; the shell membrane was exposed in the incubator for 2 hours, then sealed with sterile egg-shell and wax. As a control to these the window was made, but the membrane not exposed for 2 hours, shell and wax being at once added. This set was designed to contrast the effects of making the window, with the effects of exposing the membrane to the air in the incubator. The eggs were killed on the 13th day. The following histological findings give the results obtained:

HISTOLOGICAL FINDINGS: EXPERIMENT 1.

(*a*) Window made on 10th day and not exposed to the air: 3 normals and 1 slight hypertrophy of ectoderm and mesoderm.

(*b*) Window made on 10th day and exposed to the air for 2 hours: 5 normals and 1 hypertrophy of ectoderm; 6 hypertrophy of ectoderm, mesoderm and endoderm; 1 membrane dead and necrosed (simulating atrophy).

These results show that whilst cutting the window without exposure gave one abnormal allantoic membrane in four experiments, the additional experimental procedure of exposing the membrane in the incubator for 2 hours gave 8 abnormals in 13 experiments.

In order to further control these two factors in a second set of experiments, the window was made on the 6th day, on the side of the egg, a little distant from the embryo at a time when there was no allantoic membrane beneath the shell, and was at once sealed over with egg-shell and wax; thus any direct injury to the membrane during the making of the window was avoided.

On the 10th day, in half the eggs, the shell-covering was removed, and the window exposed to the air in the incubator for 2 hours. In the other eggs the shell was not removed. Finally the eggs were opened, and the allantoic

membrane examined on the 13th day. The following histological findings give the results obtained :

HISTOLOGICAL FINDINGS : EXPERIMENT 2.

(*a*) Window made on 6th day, not exposed : 6 normals.

(*b*) Window made on 6th day, exposed to the air for 2 hours on 10th day : 3 hypertrophy of ectoderm ; 2 hypertrophy of ectoderm, mesoderm and endoderm.

The results show that cutting the shell on the 6th day and at once sealing the hole does not injure the membrane ; on the other hand, exposing the window on the 10th day for 2 hours resulted in 5 abnormal membranes in 5 cases. It is evident, therefore, that exposing the membrane to the air in the incubator is deleterious.

In a number of cases the presence of fungi and of spore-bearing bacteria were found associated with abnormal allantoic membranes, though these could not be demonstrated in all cases. The effect of treating the eggs aseptically was therefore tried, and the following technique was adopted. Before incubation the eggs were thoroughly scrubbed with soap and water, then washed with boiled water and dried with a sterile towel ; the incubator was swabbed out with lysol. After 6 days' incubation the window was cut, the manipulation being carried out in an aseptic chamber. On the 10th day the window was removed and the egg placed in a sterile tin containing sterile wool moistened with sterile water, the tin having a thin mica window, through which radiation could be applied if required. After 2 hours the egg was removed from the tin, the window sealed down with egg-shell in the aseptic chamber, and incubated for 3 days, when it was opened and examined.

Thirteen eggs have been thus treated. In every case the allantoic membrane was normal, apart from petechial hæmorrhages in one case and an œdematous swelling in another, in which small cracks were present in the egg-shell in both cases. It is therefore concluded that this aseptic method of exposing the allantoic membrane is a reliable technique.

DISCUSSION.

Two important facts emerge from these findings : First, that changes in the allantoic membrane, exactly similar to those described by Moppett (1931) following radiation, occur in eggs dealt with by the usual technique, but not exposed to radiation. This shows that it is necessary, when dealing with this membrane, to have very carefully designed controls.

We notice in Moppett's experiments a difference between the experimentals and the controls. The experimental eggs were placed in a box with a window through which the X-rays were applied, whereas the controls were placed in a box without a window (*vide* 1931, fig. 1).

The second consideration of importance is that all the effects appear to be the result of injury. Moppett (1931) describes the reaction to X-radiation as being either atrophic or hypertrophic, as though they were two opposite

reactions; that in one case the cells were made to atrophy, and in the other, to hypertrophy. We do not take this view in respect of the reactions described, and they do not appear to differ from the reactions described by Moppett (1931); we take the view that in all cases the primary action is injurious, and that the hypertrophies which were seen are secondary to injury, and, indeed, attempts on the part of the chorio-allantoic membrane to repair damage.

SUMMARY.

A technique is described for the removal of the shell, and thus for the exposure of the shell membrane without the production of histological changes in the chorio-allantoic membrane of the chick embryo, so as to make it available for experimental purposes.

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REFERENCE.

MOPPETT, W.—(1931) *J. Cancer Res. Sydney*, **2**, 192.

THE SERUM CALCIUM CONTENT IN ECLAMPSIA.

DAVID FYFE ANDERSON,

McCunn Research Scholar.

From the Royal Maternity and Women's Hospital, and the Biochemical Laboratory of the Royal Infirmary, Glasgow.

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IT is generally agreed that during the eighth and ninth months of pregnancy the fœtus requires increasingly greater quantities of calcium (Bar, 1907). De Wesselow (1922) found that during the last months of pregnancy there is a tendency to a fall in the calcium content of the blood-serum. Bogert and Plass (1923) made observations on 23 women at the time of labour, and found that the average calcium content of the blood-serum was 9·1 mgm. per 100 c.c., while for 12 normal non-pregnant women, the average figure was 10·2 mgm. per 100 c.c. The calcium content of the blood at different stages of pregnancy

has also been studied by Widdows (1923). She has shown that, in almost all cases, there is a decrease of the calcium content towards the last months of gestation, and a general rise directly after confinement. As regards the serum calcium in cases of eclampsia, contradictory statements have been made. Stander and his co-workers (1925), while noting a tendency toward a slight lowering of the calcium in pregnancy, found no deviation from these values in nephritic toxæmia, pre-eclampsia or eclampsia. Nixon (1931) records two cases of eclampsia in which the serum calcium was respectively 8·5 and 11·3 mgm. per 100 c.c. He also gives results of serum calcium estimations in 8 cases of pre-eclampsia and 1 of hyperemesis gravidarum, and concludes that, in pregnancy toxæmia, there is little deviation from the normal value, while, in eclampsia, there may be fairly wide variations.

It was deemed expedient, therefore, to inquire into the calcium level of the blood-serum in the eclamptic state and other allied conditions.

METHOD.

The method adopted was that of Kramer and Tisdall (1921), as modified by Clark and Collip (1925), by which the normal serum calcium is found to vary between 9·0 and 11·0 mgm. per 100 c.c. Stewart and Dunlop (1930) give the normal range of serum calcium as 9·5–10·5 mgm. per 100 c.c. Many of the estimations here recorded were done in duplicate in order to test the accuracy of the method. Greville (1931) has recently reported that the method gives an accurate measure of the blood-calcium. The results are shown in Tables I to IV. In Table V are given notes of the cases of eclampsia treated by injection of calcium gluconate.

TABLE I.—*Cases of Normal Pregnancy at Full Time, in Labour and Shortly after Delivery, Grouped according to Serum Calcium Value (mgm. per 100 c.c. Serum) (35 Cases).*

Levels of serum Ca mgm. per 100 c.c. serum.	Number of cases.	Approximate percentage of total.	Remarks.
12·1–12·5	..	..	..
11·6–12·0	1	2·9	..
11·1–11·5	..	..	..
10·6–11·0	1	2·9	..
10·1–10·5	7	20·0	..
9·6–10·0	7	20·0	..
9·1–9·5	13	37·2	..
8·6–9·0	5	14·3	..
8·1–8·5	1	2·9	Post-partum hæmorrhage.

Approximately 17 per cent. show values of 9 mgm. per 100 c.c. and under (taking 9 mgm. per 100 c.c. as the lower limit of normality).

TABLE II.—*Cases of Nephritic and Pre-eclamptic Toxæmia Grouped according to Serum Calcium Value* (17 *Cases*).

Levels of serum Ca mgm. per 100 c.c. serum.	Number of cases.	Approximate percentage of total.
12·1–12·5	1	5·9
11·6–12·0	..	..
11·1–11·5	..	..
10·6–11·0	..	..
10·1–10·5	..	..
9·6–10·0	2	11·8
9·1–9·5	4	23·6
8·6–9·0	2	11·8
8·1–8·5	3	17·7
7·6–8·0	2	11·8
7·1–7·5	3	17·7

Taking 9·0 mgm. per 100 c.c. as the lower limit of normality, approximately 59 per cent. of these cases show subnormal values.

TABLE III.—*Cases of Eclampsia Grouped according to Serum Calcium Value* (44 *Cases*).

Levels of serum Ca mgm. per 100 c.c. serum.	Number of cases.	Approximate percentage of total.	Remarks.
11·1–11·5	1	2·3	3 minutes before sixteenth and last fit.
10·6–11·0	..	..	..
10·1–10·5	..	..	..
9·6–10·0	6	13·6	In 1 case 10 minutes after last fit.
9·1–9·5	1	2·3	After second and last fit.
8·6–9·0	5	11·3	..
8·1–8·5	12	27·2	..
7·6–8·0	13	29·5	In 1 case later estimation gave value of 5·7.
7·1–7·5	4	9·0	..
6·6–7·0	1	2·3	..
6·1–6·5	1	2·3	..

In approximately 82 per cent. of these cases of eclampsia the serum calcium value is subnormal.

DISCUSSION.

In the present paper no account has been taken of the two forms—diffusible and non-diffusible calcium—as it was considered that the main object was to establish whether there was or was not a definite lowering of the serum calcium

due to this toxæmia, eclampsia. Further, it was held that present methods are not sufficiently reliable to warrant the determination of the partition, although work has been attempted in this field by (among others) Bokelmann and Bock (1928), using the method of Rona, Haurowitz and Petow.

The very abrupt increase in calcium retention by the fœtus, which was shown by Bar (1907) to occur in the last sixty days of pregnancy, must mean a sudden change in the demands made upon the maternal stores of calcium. The

TABLE IV.—*Average Value of Serum Calcium in Various Conditions not Previously Mentioned* (31 *Cases*).

Average serum Ca mgm. per 100 c.c. serum.	Type of case.	Number of cases.
4·1	Toxic jaundice ; fœtus macerated ; placenta $3\frac{3}{4}$ lb. ; death	1
8·2	Puerperal sepsis	1
8·9	Repeated abortions	1
9·0	Uræmia ; polycystic kidneys	1
9·1	Normal puerperium ; second day	1
9·2	Pyelitis	4
lowest 9·0, highest 9·2		
9·2	Chorea gravidarum	3
lowest 8·8, highest 9·6		
9·3	Hysteria	2
lower 9·1, higher 9·5		
9·4	Hyperemesis gravidarum	7
lowest 8·1, highest 11·2		
9·5	Jacksonian epilepsy	1
9·6	Twin pregnancy shortly after delivery	1
9·8	Incomplete abortion	3
lowest 9·5, highest 10·1		
10·0	Ante-partum hæmorrhage	2
10·0	Pyelitis in labour	1
10·5	Rheumatoid arthritis	1
12·5	Exophthalmic goitre	1

maternal blood contains about 0·5 gm. calcium, and the requirements of the fœtus necessitate the supply of approximately 8 gm. calcium during the last month of pregnancy (Theobald, 1930). Unless the pregnant and lactating woman be provided with adequate supplies of calcium, the drain of calcium into the fœtus directly and through the milk secretion is liable to be followed by untoward results.

In the present investigation, 82 per cent. of the cases of eclampsia had serum calcium values below 9 mgm. per 100 c.c. As will be seen from the other tables, the lowering of the serum calcium value is not peculiar to eclampsia, but, relative to pre-eclamptic and other toxæmic conditions, the proportion of

cases showing a low value is much greater. Of 20 normal cases in labour, the average serum calcium value was found to be 9·9 mgm. per 100 c.c., while, in 12 normal cases, shortly after delivery, the average reading was 9·4 mgm. per 100 c.c.

TABLE V.—*Results of Treatment with Calcium Gluconate* (10 *c.c.* 10 *per cent. Solution*) *in Eclampsia.*

Case number.	Method of administration.	Number of fits.	Remarks.
1 (3-gravida)	5 c.c. subcutaneously 5 c.c. intravenously	6	No obvious effect; died undelivered. Liver fatty, with much punctate subcapsular hæmorrhage. Lungs œdematous, large recent infarctions. Myocardium fatty and very soft. Brain moderate œdema. Uterus showed retroplacental clot. Placenta very degenerate, with infarctions, both recent and of long standing.
2 (1-gravida)	10 c.c. subcutaneously	15	8 fits after injection before forceps delivery of dead child; 1 fit post-partum. Developed mental symptoms, but ultimately recovered.
3 (5-gravida)	10 c.c. intravenously	7	1 fit after injection. About 7 hours later spontaneous delivery. Slight mental symptoms. Blood-pressure remained high (over 200 mm. Hg.) for some days. 1 month after delivery B.P. 150 mm. Hg. Serum Ca before injection was 8·8 mgm. per 100 c.c.; 15 days later it was 10·2 mgm. per 100 c.c.
4 (1-gravida)	10 c.c. intravenously	10	4 fits before forceps delivery of mature live child, 6 thereafter. Injection given after sixth post-partum fit; no further fits. Uninterrupted recovery. Before injection, serum Ca was 7·9 mgm. per 100 c.c.; 22 days later it was 11·6 mgm. per 100 c.c.
5 (2-gravida)	10 c.c. intravenously	9	6 fits after injection. Os undilated. Death 12 hours after first fit. Undelivered.
6 (1-gravida)	10 c.c. intramuscularly	3	No further fits after injection. Premature live child delivered by Cæsarian section. Uninterrupted recovery.
7 (1-gravida)	10 c.c. intramuscularly on 2 occasions	2	1 fit after first injection. Mature live child delivered by forceps. Recovery.
8 (1-gravida)	10 c.c. intramuscularly on 2 occasions	14	8 fits after first injection; none after second. 2 fits just before forceps delivery; 12 fits thereafter. Mental symptoms during puerperium, but complete recovery. Serum Ca when in labour before onset of fits = 9·9 mgm. per 100 c.c. On sixth day of puerperium serum Ca = 9·7 mgm. per 100 c.c.

The milk fever of cows presents very similar symptoms to those found to be associated with the lowered calcium content of the blood of parathyroidectomized animals, and it has been related to eclampsia in the human. Little and Wright (1925) investigated a suggestion made by Dryerre and Greig (1921), and showed that the onset of milk fever is accompanied by a greatly

reduced concentration of calcium, the severity of the case being paralleled by a proportionately large decrease in calcium concentration. In the treatment of milk fever, inflation of the mammary gland has been practised for many years ; gaseous inflation or the injection of fluid is known as a specific for milk fever in cattle. Greig (1930) found that the effect of this procedure is to bring about a rise in the blood-calcium values, this rise being coincident with and bearing a direct relation to the disappearance of the symptoms of the disease. From a further study of such cases, in which he found milk fever invariably to be associated with a very low blood-calcium content, Greig suggested acute calcium deficiency as the essential cause of milk fever. Prophylaxis, by inclusion of calcium in the diet, has been advocated, but, as Greig has pointed out, many cows with milk fever have received an abundance of calcium in their food. The injection of parathyroid extract, with a view to converting the deposited calcium in bone into an ionized and readily assimilable form, is found in practice to be an inadequate method of treatment. A readily assimilable salt of calcium, such as the gluconate, was advocated by Greig as a practicable measure in the treatment of milk fever.

In a case of eclampsia, Minot and Cutler (1929) found prompt symptomatic relief to follow the intravenous injection of 10 c.c. of a 10 per cent. solution of calcium gluconate.

With regard to the treatment of eclampsia by administration of calcium, it is clear from the few cases observed in this series that a single injection of 10 c.c. of a 10 per cent. solution of calcium gluconate is not sufficient to cause the immediate cessation of fits, even if given intravenously. The persistence of fits after injection might be accounted for by the fact that a certain amount of time must elapse before maximal elevation of the serum calcium can occur. Further injections would probably yield more dramatic results, but caution is evidently necessary in repeating the dose, especially if given by the intravenous route (*vide* Lieberman, 1931).

CONCLUSIONS.

The lowering of the serum calcium in such a large percentage of cases of eclampsia and pre-eclampsia is suggestive. With a lessened concentration, as occurs towards the end of pregnancy, any sudden change or demand on the reserves may prove too much for the maternal organism. It is not indicated that lowered calcium concentration is necessarily the ætiological factor in eclampsia, but is a sign, just as is the raised serum inorganic sulphur (Anderson and Tompsett, 1932). Calcium deficiency may not be due to an absence of calcium from the body, but to the incapacity to utilize the calcium which is present. Symptoms which appear in normal pregnancy, due to the increased activity of the organs of internal secretion, may reach a degree which is definitely pathological in an organism less capable of adaptation to the changing demands of the fœtal organsim. It is a question of supply and demand, of adaptability or failure.

SUMMARY.

1. 82 per cent. of the 44 cases of eclampsia investigated showed subnormal serum calcium values—that is, 9 mgm. per 100 c.c. and under.

2. 59 per cent. of the 17 cases of nephritic and pre-eclamptic toxæmia were associated with a subnormal serum calcium.

3. 17 per cent. of the 35 cases of normal pregnancy at full time, in labour, and shortly after delivery had serum calcium values lower than normal.

4. 31 other cases, mainly of complicated pregnancy, were examined in respect of their serum calcium level.

5. From the available data, it is impossible to assess the effect of injections of calcium gluconate, intravenously or otherwise. In the doses given—10 c.c. —it has no abrupt effect on the frequency of the fits.

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REFERENCES.

Anderson, D. F., and Tompsett, S. L.—(1932) *Brit. J. Exp. Path.*, **13**, 130.
Bar, P.—(1907) 'Léçons de Pathologie Obstétricale,' **2**, 446, Paris (Asselin et Houzeau).
Bogert, L. J., and Plass, E. D.—(1923) *J. Biol. Chem.*, **56**, 297.
Bokelmann, O., and Bock, A.—(1928) *Arch. Gynäk.*, **133**, 308 and 739.
Clark, E. P., and Collip, J. B.—(1925) *J. Biol. Chem.*, **63**, 462.
Dryerre, H., and Greig, J. R.—(1921) *Ibid.*, **49**, 487.
Greig, J. R.—(1930) *Vet. Rec.*, **10**, 115.
Greville, G. R.—(1931) *Biochem. J.*, **25**, 1931.
Kramer, B., and Tisdall, F. F.—(1921) *J. Biol. Chem.*, **47**, 475.
Lieberman, A. L.—(1931) *J. Amer. Med. Ass.*, **97**, 15.
Little, W. L., and Wright, N. C.—(1925) *Brit. J. Exp. Path.*, **6**, 129.
Minot, A. S., and Cutler, J. T.—(1929) *Proc. Soc. Exp. Biol.*, **26**, 607.
Nixon, W. C. W.—(1931) *Lancet*, **2**, 291.
Stander, H. J., Duncan, E. E., and Sisson, W. E.—(1925) *Johns Hopk. Hosp. Bull.*, **36**, 411.
Stewart, C. P., and Dunlop, D. M.—(1930) *Clinical Chemistry in Practical Medicine*, p. 225 (E. & S. Livingstone, Edinburgh).
Theobald, G. W.—(1930) *Lancet*, **1**, 1115.
de Wesselow, O. L. V.—(1922) *Ibid.*, **2**, 227.
Widdows, S. T.—(1923) *Biochem. J.*, **17**, 34.